1 of 3

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1. WO 04/00153, "VACCINE COMPOSITION CONTAINING ADJUVANTS", International Filing Date: 15 June 1993.

2. U.S. Patent No. 5,057,540.

3. U.S. Patent No. 4,912,094.

4. Li et al., 5, *J. of Acquired Immune Deficiency Syndromes*, No. 7 (1992).

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16. Advisory Action, 2/23/99.

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(54) Title: VACCINE COMPOSITION CONTAINING ADJUVANTS

(57) Abstract

The present invention provides vaccine compositions comprising 3 De-O-acylated monophosphoryl lipid A and QS21. The vaccines compositions are potent inducers of CTL and γ IFN responses.

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VACCINE COMPOSITION CONTAINING ADJUVANTS

The present invention relates to novel vaccine formulations, to methods of their production and to their use in medicine. In particular, the present invention relates to vaccines containing QS21, an Hplc purified non-toxic fraction derived from the bark of Quillaja Saponaria Molina, and 3 De-O-acylated monophosphoryl lipid A (3 D-MPL).

3 De-O-acylated monophosphoryl lipid A is known from GB2220 211 (Ribi), Chemically it is a mixture of 3-deacylated monophosphoryl lipid A with 4, 5 or 6 acylated chains and is manufactured by Ribi Immunochem Montana.

QS21 is a Hplc purified non toxic fraction of a saponin from the bark of the South American tree Quillaja saponaria molina and its method of its production is disclosed (as QA21) in US patent No. 5,057,540.

The present invention is based on the surprising discovery that formulations containing combinations of QS21 and 3 D-MPL synergistically enhance immune responses to a given antigen.

For example a vaccine formulation of the malarial antigen, RTS, S in combination with 3D-MPL and QS21 results in a powerful synergistic induction of CS protein specific cytotoxic T lymphocyte (CTL) response in the spleen.

RTS is a hybrid protein comprising substantially all the C-terminal portion of the circumsporozoite (CS) protein of P.falciparum linked via four amino acids of the $preS_2$ portion of Hepatitis B surface antigen to the surface (S) antigen of hepatitis B virus. It's full structure is disclosed in co-pending International Patent Application No. PCT/EP92/02591, published under Number WO 93/10152 claiming priority from UK patent application No.9124390.7. When expressed in yeast RTS is produced as a lipoprotein particle, and when it is co-expressed with the S antigen from HBV it produces a mixed particle known as RTS,S.

The observation that is possible to induce strong cytolytic T lymphocyte responses is significant as these responses, in certain animal models have been shown to induce protection against disease.

The present inventors have shown that the combination of the two adjuvants QS21 and 3D-MPL with the recombinant particulate antigen RTS,S results in a powerful induction of CS protein specific CTL in the spleen. QS21 also enhances induction of CTL on its own, while 3D-MPL does not. The combination can be said to act in a synergistic way, because it has an effect that is larger than the sum of the separate effects of each adjuvant. The synergy between these two adjuvants for CTL induction is a surprising observation which has important implications for the use of recombinant molecules as vaccines for induction of CTL mediated immunity.

Induction of CTL is easily seen when the target antigen is synthesised intracellularly (e.g. in infections by viruses, intracellular bacteria, or in tumours), because peptides generated by proteolytic breakdown of the antigen can enter the appropriate processing pathway, leading to presentation in association with class I molecules on the cell membrane. However, in general, pre-formed soluble antigen does not reach this processing and presentation pathway, and does not elicit class I restricted CTL. Therefore conventional non-living vaccines, while eliciting antibody and T helper responses, do not generally induce CTL mediated immunity. The combination of the two adjuvants QS21 and 3D-MPL can overcome this serious limitation of vaccines based or recombinant proteins, and induce a wider spectrum of immune responses.

CTL specific for CS protein have been shown to protect from malaria in mouse model systems (Romero et al. Nature 341:323 (1989)). In human trials where volunteers were immunised using irradiated sporozoites of P. falciparum, and shown to be protected against subsequent malaria challenge, induction of CTL specific for CS epitopes was demonstrated (Malik et al. Proc. Natl. Acad. Sci. USA 88:3300 (1991)).

The ability to induce CTL specific for an antigen administered as a recombinant molecules is relevant to malaria vaccine development, since

the use of irradiated sporozoites would be impractical, on the grounds of production and the nature of the immune response.

In addition to malaria vaccines, the ability to induce CTL responses would benefit vaccines against herpes simplex virus, cytomegalovirus, human Immunodeficiency virus, and generally all cases where the pathogen has an intracellular life stage.

Likewise, CTL specific for known tumour antigens could be induced by a combination of a recombinant tumour antigen and the two adjuvants. This would allow the development of anti cancer vaccines.

In certain systems, the combination of 3D-MPL and QS21 have been able to synergistically enhance interferon γ production. The present inventors have demonstrated the synergistic potential of 3D-MPL and QS21 by utilising a herpes simplex antigen known as gD_2t. gD_2t is a soluble truncated glycoprotein D from HSV-2 and is produced in CHO cells according to the methodology Berman et al. Science 222 524-527.

IFN-γ secretion is associated with protective responses against intracellular pathogens, including parasites, bacteria and viruses. Activation of macrophages by IFN-γ enhances intracellular killing of microbes and increases expression of Fc receptors. Direct cytotoxicity may also occur, especially in synergism with lymphotoxin (another product of TH1 cells). IFN-γ is also both an inducer and a product of NK cells, which are major innate effectors of protection. TH1 type responses, either through IFN-γ or other mechanisms, provide preferential help for IgG2a immunoglobulin isotypes.

Glycoprotein D is located on the viral envelope, and is also found in the cytoplasm of infected cells (Eisenberg R.J. et al J. of Virol. 1980 35 428-435). It comprises 393 amino acids including a signal peptide and has a molecular weight of approximately 60kD. Of all the HSV envelope glycoproteins this is probably the best characterized (Cohen et al. J. Virology 60 157-166). In vivo it is known to play a central role in viral attachment to cell membranes. Moreover, glycoprotein D has been shown to be able to elict neutralizing antibodies in vivo (Eing et al, J. Med Virology 127: 59-65). However, latent HSV2 virus can still be reactivated

and induce recurrence of the disease despite the presence of high neutralizing antibodies titre in the patients sera. It is therefore apparent that the ability to induce neutralizing antibody alone is insufficient to adequately control the disease.

In order to prevent recurrence of the disease, any vaccine will need to stimulate not only neutralizing antibody, but also cellular immunity mediated through T-cells, particularly cytotoxic T-cells.

In this instance the gD_2t is HSV2 glycoprotein D of 308 amino acids which comprises amino acids 1 though 306 of the naturally occurring glycoprotein with the addition of Asparagine and Glutamine at the C terminal end of the truncated protein. This form of the protein includes the signal peptide which is cleaved to yield a mature 283 amino acid protein. The production of such a protein in Chinese Hamster ovary cells has been described in Genentech's European patent EP-B-139 417.

The mature truncated glycoprotein D (rgD2t) or equivalent proteins secreted from mammalian cells, is preferably used in the vaccine formulations of the present invention.

The formulations of the present invention are very effective in inducing protective immunity in a genital herpes model in guinea pigs. Even with very low doses of antigen (e.g. as low as 5 μg rgD2t) the formulations protect guinea pigs against primary infection and also stimulate specific neutralising antibody responses. The inventors, utilising formulation of the present invention, have also demonstrated Effector cell mediated responses of the TH1 type in mice.

Accordingly, the present invention provides a vaccine or pharmaceutical formulation comprising an antigen in conjunction with 3 Deacylated monophosphoryl lipid A and QS21. Such a formulation is suitable for a broad range of monovalent or polyvalent vaccines.

Preferably the vaccine formulations will contain an antigen or antigenic composition capable of eliciting an immune response against a human or animal pathogen, which antigen or antigenic composition is derived from HIV-1, (such as gp120 or gp160), any of Feline Immunodeficiency virus,

human or animal herpes viruses, such as gD or derivatives thereof or Immediate Early protein such as ICP27 from HSV1 or HSV2, cytomegalovirus ((esp Human)(such as gB or derivatives thereof), Varicella Zoster Virus (such as gpI, II or III), or from a hepatitis virus such as hepatitis B virus for example Hepatitis B Surface antigen or a derivative thereof, hepatitis A virus, hepatitis C virus and hepatitis E virus, or from other viral pathogens, such as Respiratory Syncytial virus, human papilloma virus or Influenza virus, or derived from bacterial pathogens such as Salmonella, Neisseria, Borrelia (for example OspA or OspB or derivatives thereof), or Chlamydia, or Bordetella for example P.69, PT and FHA, or derived from parasites such as plasmodium or Toxoplasma.

The formulations may also contain an anti-tumour antigen and be useful for immunotherapeutically treating cancers.

The formulation may also be useful for utilising with herpetic light particles such as described in International Patent Application No. PCT/GB92/00824 and, International Patent Application No. PCT/GB92/00179.

Derivatives of Hepatitis B Surface antigen are well known in the art and include, inter alia, those PreS$_1$, PreS$_2$ S antigens set forth described in European Patent applications EP-A-414 374; EP-A-0304 578, and EP 198-474.

In a further aspect of the present invention there is provided a vaccine as herein described for use in medicine.

The ratio of QS21 : 3D-MPL will typically be in the order of 1 : 10 to 10 : 1; preferably 1 : 5 to 5 : 1 and often substantially 1 : 1. The preferred range for optimal synergy is 2.5:1 to 1:1 3D MPL: QS21. Typically for human administration QS21 and 3D MPL will be present in a vaccine in the range 1 µg - 100 µg, preferably 10 µg - 50 µg per dose. Often the vaccine will not require any specific carrier and be formulated in an aqueous or other pharmaceutically acceptable buffer. In some cases it may be advantageous that the vaccines of the present invention will further contain alum or be presented in an oil in water emulsion, or other suitable

vehicle, such as for example, liposomes, microspheres or encapsulated antigen particles.

Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Maryland, U.S.A. 1978. Encapsulation within liposomes is described, for example, by Fullerton, U.S. Patent 4,235,877. Conjugation of proteins to macromolecules is disclosed, for example, by Likhite, U.S. Patent 4,372,945 and by Armor et al., U.S. Patent 4,474,757.

The amount of protein in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccinees. Such amount will vary depending upon which specific immunogen is employed and how it is presented. Generally, it is expected that each dose will comprise 1-1000 μg of protein, preferably 2-100 μg, most preferably 4-40 μg. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. Following an initial vaccination, subjects may receive one or several booster immunisation adequately spaced.

The formulations of the present invention maybe used for both prophylatic and therapeutic purposes.

Accordingly in one aspect, the invention provides a method of treatment comprising administering an effective amount of a vaccine of the present invention to a patient.

Examples

1.0 Synergy between 3D MPL and QS21 for induction of Interferon γ secretion.

In order to test the ability of 3D MPL and QS21 based adjuvant formulations of rgD2t, to induce effector cell mediated immune responses, groups of Balb/c mice were vaccinated, and their draining lymph node cells tested for IFN-γ secretion as described below.

1.1 rgD2t formulations

This experiment compared three adjuvant formulations:

i) rgD2t in 3D-MPL
ii) rgD2t in QS21
iii) rgD2t in 3D-MPL/QS21

These formulations were made up as follows. rgD2t was produced in CHO cells and corresponds to the mature 1-283 amino acids of HSV-2 gD and is produced according to the methodology of Berman (supra) and EP 0139417.

* rgD2t / 3D-MPL

5 µg of rgD2t/dose are incubated 1h, under agitation,at room temperature, then mixed with a 3D-MPL suspension (25 µg/dose). The volume is adjusted to 70 µl/dose using a sodium chloride solution (5M, pH 6.5 ± 0.5) and water for injection to obtain a final concentration of 0.15M sodium chloride. pH is kept at 6.5 ± 0.5.

* rgD2t/QS21

5 µg rgD2t/dose are incubated 1h at room temperature under agitation The volume is adjusted using sodium chloride solution (5M, pH 6.5 ± 0.5) and water for injection to 70µl. QS21 (10 µg/dose) is then added. pH is kept at 6.5 ± 0.5.and sodium chloride final concentration at 0.15M.

* rgD2t/3D-MPL / QS21.

5 µg rgD2t/dose are incubated 1h at room temperature under agitation. 3D-MPL (25 µg/dose) is added as an aqueous suspension. The final volume of 70µl is completed by addition of an aqueous solution of QS21 (10 µg/dose) and the pH kept at 6.5 ± 0.5 and the sodium chloride concentration at 0.15M.

1.2 IMMUNISATION

Mice were injected into the hind footpads with 35 µL/footpad of formulation. Thus each mouse received 70 µL. Immunisation were on days 0, and 14. Animals were sacrificed on day 21.

1.3 INTERFERON γ ASSAYS

Popliteal lymph node cells from immunised mice were stimulated in vitro using rgD2t at 10, 1, 0.1, 0 µg/ml. Triplicate cultures (200 µl volumes) were set up in round bottom 96-well microtiter plates, using 2 x 10^5 responder cells and 2 x 10^5 irradiated (3000 rad) syngeneic naive spleen cells. Culture medium was RPMI 1640 with 10% foetal calf serum. Aliquots of 100 µl of culture medium from each replicate were harvested and pooled for IFN-γ determinations. Cultures were assayed at 72 hours. For all assays, a control group using ConA (Boehringer Mannheim) at 5 µg/mL was included. This was always positive.

Secretion of IFN-γ was determined using a commercial ELISA assay manufactured by Holland Biotechnology (distributed by Gibco). Assays were carried out on 100 µl of pooled supernatant from triplicate wells.

Secretion of IFN-γ above the assay background of 50 pg/µl was observed in all three formulation groups (see Table). In addition, a synergistic effect between QS21 and 3D-MPL was observed. While each adjuvant on its own induced cells capable of secreting IFN-γ in response to rgD2t, their combination induced more than twice the sum of individual responses.

1.4 Results

Synergy between QS21 and 3D-MPL for induction of IFN-γ secretion.

Immunization:		QS21/3D-MPL rgD2t	QS21 rgD2t	3D-MPL rgD2t
In vitro	10.0	1351	1105	515
stimulation	1.0	914	116	192
(μg/mL gD2t):	0.1	335	<50	143
	0.0	101	<50	139

IFN-γ is expressed in pg/mL.

The table clearly shows that the combined vaccine induces IFN-γ secretion in a synergistic manner.

2.0 Synergy Between 3D MPL and QS21 for the induction of CTLs

In order to test the ability of RTS,S particles in 3D MPL and QS21 based adjuvant formulations to induce CTLs, groups of B10.BR mice were immunised and their spleen cells stimulated in vitro and tested in cytotoxicity assays on L cells expressing the CS protein.

2.1 Formulation of RTS,S particles.

RTS,S particles were formulated in three different compositions:

1. RTS,S particles ((10μg) with QS21 (10μg) and 3D-MPL (25μg);
2. RTS,S particles ((10μg) with QS21 (10μg);
3. RTS,S particles ((10μg) with 3D-MPL (25μg);

The formulations were made up as follows:

RTS, S/3 D MPL

10 µg of RTS, S particles/dose was incubated at room temperature under agitation then mixed with a 3D MPL aqueous suspension (25µg/dose). The volume is then adjusted to 70 µl/dose using water for injections and a sodium chloride solution (5N, pH 6.5 ± 0.5) to reach a final concentration of 0.15M sodium chloride (pH is kept at 6.5 ± 0.5).

RTS,S /QS21

10µg of RTS, S particles/dose incubated 1h. at room temperature under agitation. The volume is adjusted using water for injection and a sodium chloride solution (5N, pH 6.5 ± 05) and completed to a final volume of 70µ l/ dose with an aqueous solution of QS21 (10µg/dose). pH is kept at 6.5 ± 0.5 and sodium chloride final concentration at 0.15M.

RTS,S / 3 D MPL / QS21

10 µg of RTS,S particles / dose are incubated 1h. at room teperature under agitation then mixed with a 3D MPL (aqueous suspension (25µg/dose) - The volume is then adjusted with water for injection and a sodium chloride solution (5D pH 6.5 ± 0.5). The final volume is completed by addition of an aqueous solution of QS21 (10µg/dose). pH is kept at 6.5 ± 0.5, and sodium chloride final concentration at 0.15 M.

2.2 Immunisation of mice with RTS,S particles

Four to six week old female mice of the strain B10.BR (H-2^k) were purchased from IFFA CREDO (France). Groups of 3 animals were immunised by intra foot-pad injection of 35 µL of antigen formulation into each hind limb. The animals were boosted with a second equal dose of antigen injected two weeks later.

2.3. In vitro stimulation on anti CS CTL

Two weeks after the boost, spleen cells were harvested and stimulated in vitro using syngeneic fibroblasts transfected with the P. falciparum circumsporozoite protein gene (7G8 clone). These CS-transfectant cells

have been described in the paper by Kumar, S. et al. (1988), Nature 334:258-260.

The cultures were established in RPMI 1640 medium supplemented with 10% of heat inactivated foetal calf serum and usual additives, in conditions well known to those of skill in the art.

Responder cells were cultured at a concentration of 10^6 cells/mL in the presence of 10^5 CS-transfectants per mL. To prevent proliferation of CS-transfectant cells, these were irradiated using a dose of 2×10^4 rad. The cultures were fed by replacing 1/2 of culture medium on day 3 and 6, and tested for cytolytic activity on day 7.

2.4. Cytotoxicity assay for anti-CS CTL

Responder cell cultures were harvested, washed, and mixed at ratios varying from 100:1 to 0.3:1 with a constant number of 2000 target cells, in volumes of 200 µL of medium in V-bottom 96-well plates.
Target cells were syngeneic fibroblast cells that had been labelled with ^{51}Cr.

Two different types of target cells were used:

1. L cells
2. CS transfected L cells

These are described in: Kumar, S. et al. (1988), Nature 334:258-260.

The assay was incubated for 6 hours at 37°C, then the amount of radioactivity released into the supematant by lysis of target cells was determined. Cytolytic activity is expressed as % specific lysis:

Results:

Target cells:	Effector: target ratio	% Specific lysis by formulation: 1. RTS,S/ QS21/ 3D-MPL	2. RTS,S/ QS21/	3. RTS,S/ 3D-MPL
CS transfected L cells	100	58	17	1
	30	53	10	0
	10	47	5	1
	3	27	1	0
	1	11	0	0
	0.3	2	-2	-1
L cell	100	3	-2	5
	30	-2	1	4
	10	0	-1	2
	3	0	3	4
	1	-1	4	2
	0.3	3	1	2

Immunisation of B10.BR mice with RTS,S adjuvanted with QS21 and 3D-MPL (formulation #1) induced in the spleen high levels of CTL specific for the circumsporozoite component of RTS,S. Immunisation with RTS,S particles adjuvanted with QS21 (formulation #2) also induced CTL in the spleen, but only at about 1/30th of the levels given by formulation #1. RTS,S with 3D-MPL (formulation #3) did not induce CTL.

Since the target cells used in this assay do not express MHC class II molecules, the effector cells can be assumed to be $CD8^+$, class I restricted CTL.

3. Other formulation

Hepatitis B Surface Antigen, Alum 3D-MPL and QS21.

The preparation of Hepatitis B Surface antigen (HBsAg) is well documented. See for example Harford et al Develop. Biol. Standard 54 p125 (1983), Gregg et al Biotechnology 5 p479 (1987) EP-A-O 226 846 and EP-A-299 108 and references therein.

3D-MPL was obtained from Ribi Immunochem, QS21 was obtained from Cambridge Biotech, and Aluminium hydroxide was obtained from Superfos (Alhydrogel).

A number of different formulations were made up for studies of cell mediated immunity in mice and for studies in Rhesus monkeys.

3.1 Formulation 1 was made up in phosphate buffer (pH 6.8) to comprise the following per 60 µl dose.

20 µg	HBsAg
30 µg	$Al(OH)_3$
30 µg	3D - MPL
10 µg	QS 21
10 mM	PO_4^{3-}
0.15 M	NaCl

The formulation was made up in the following manner. 20µg HBsAg/dose was incubated with $Al(OH)_3$ for one hour at room temperature with gentle shaking. 3D-MPL was added as an aqueous suspension, and the formulation completed by the addition of QS21, phosphate buffer and sodium chloride and incubated for one hour at room temperature. The final formulation had a pH of between 6.5 and 7.0 and used for foot pad studies in mice.

3.2 Formulation 2 was made up in a phosphate buffer (pH6.8) to comprise the following per 200 µl dose.

1 µg	HBsAg
100 µg	$Al(OH)_3$
50 µg	3D-MPL
20 µg	QS 21
10 mM	PO_4^{3-}
0.15 M	NaCl

The formulation was made up in the following manner. HBsAg and $Al(OH_3)$ were incubated together for one hour at room temperature with

gentle shaking. The formulation was completed by the addition of $Al(OH)_3$, 3D-MPL as an aqueous suspension and QS21, with phosphate buffer and sodium chloride solution and incubated again for thirty minutes. The pH of the formulation was kept between 6.5 and 7.0 and used for Humoral immunity studies in mice.

3.3 Formulation 3 was made up in a similar manner, in a phosphate buffer (pH6.5 - 7.0) to contain the following per 1 ml dose :

10 µg	HBsAg
500 µg	$Al(OH)_3$
50 µg	3D-MPL
10 µg	QS 21

The formulation was used for monkey studies.

4. Conclusions

The combination of the two adjuvants QS21 and 3D-MPL with the recombinant particulate antigen RTS,S resulted in a powerful induction of CS protein specific CTL in the spleen. QS21 enhances induction of CTL on its own, while 3D-MPL does not. The combination can be said to act in a synergistic way, because it has an effect that is larger than the sum of the separate effects of each adjuvant. The synergy between these two adjuvants for CTL induction is a surprising observation which supports our observation of synergy between QS21 and 3D-MPL for induction of T cells capable of secreting IFN-γ in response to stimulation with the soluble recombinant protein gD2t. This finding has important implications for the use of recombinant molecules as vaccines for induction of CTL mediated immunity, since the combination of the two adjuvants QS21 and 3D-MPL can overcome this serious limitation of vaccines based on recombinant proteins, and induce a wider spectrum of immune responses than hitherto.

The mouse cell mediated immunogenicity data show that QS21 based formulations of rgD2t induce a significant synergistic TH1 type T cell response (IFN-γ secretion).

Such TH1 type T cells have been shown to be involved in induction of delayed type hypersensitivity responses in mice. Our own data in prophylaxis of HSV disease show that concomitant induction of neutralizing antibody titers and antigen specific DTH responses affords the best protection against herpes simplex disease.

Put together, these data suggested to us that QS21 formulations of rgD2t may be effective in inducing a protective response against HSV disease. The data presented show an unexpected synergistic effect between 3D Monophosphoryl lipid A and QS21, in inducing IFN-γ secreting antigen specific T cells. Such a synergy may translate in improved ability to induce a protective response against HSV disease, and indeed these formulations are effective in protecting against disease in guinea pigs.

Claims

1. A vaccine composition comprising an antigen and/or antigenic composition, QS21 and 3 De-O-acylated monophosphoryl lipid A (3D-MPL).

2. A vaccine as claimed in claim 1 wherein the ratio of QS21:3D-MPL is from 1:10 to 10:1.

3. A vaccine composition as claimed in claim 1 or 2 capable of invoking a cytolytic T cell response in a mammal to the antigen or antigenic composition.

4. A vaccine composition as claimed in any of claims 1 to 3 capable of stimulating interferon γ production.

5. A vaccine composition as claimed in any of claims 1 to 4 wherein the ratio of QS21:3D-MPL is from 1:1 to 1:2.5.

6. A vaccine composition as claimed herein comprising an antigen or antigenic composition derived from any of Human Immunodeficiency Virus, Feline Immunodeficiency Virus, Herpes Simplex Virus type 1, Herpes Simplex virus type 2, Human cytomegalovirus, Hepatitis A,B,C or E, Respiratory Syncytial virus, human papilloma virus, Influenza virus, Salmonella, Neisseria, Borrelia, Chlamydia, Bordetella, Plasmodium or Toxoplasma.

7. A vaccine as claimed in any of claim 1 to 5 wherein the antigen is a tumour antigen.

8. Use of composition as defined in any of claims 1 to 5 for the manufacture of a vaccine for the prophylatic treatment of viral, bacterial, or parasitic infections.

9. Use of composition as defined in any of claims 1 to 5 for the manufacture of a vaccine for the immunotherapeutic treatment of viral, bacterial, parasitic infections or cancer.

10. A method of treating a mammal suffering from or susceptible to a pathogenic infection comprising the administration of a safe and effective amount of a composition according to any of claims 1 to 5.

11. A method of treating a mammal suffering from cancer comprising the administration of a safe and effective amount of a composition according to any of claims 1 to 5.

12. A process for making a vaccine composition according to claims 1 to 5 comprising admixing QS21 and 3D-MPL with an antigen or antigenic composition.

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 93/01524

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A61K39/39; A61K39/00; // A61K39/245,A61K39/295

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	BIOTECHNOLOGY vol. 20, 1992, pages 431 - 449 ANTHONY C. ALLISON ET AL. 'IMMUNOLOGICAL ADJUVANTS AND THEIR MODE OF ACTION' see page 437, paragraph 19.6 - page 439 see page 441, paragraph 19.8 - page 442	1
A	J. ANIMAL SCIENCE vol. 68, 1990, pages 3742 - 3746 A. J. ROBERTS ET AL. 'ACTIVE IMMUNIZATION OF BEEF HEIFERS AGAINST LUTEINIZING HORMONE...' see page 3743; table 1	1

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
29 SEPTEMBER 1993	04. 10. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	REMPP G.L.E.

INTERNATIONAL SEARCH REPORT

Int. ational application No.
PCT/EP 93/01524

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark : Although claims 10-11 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Exhibit 2

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,057,540

DATED : October 15, 1991

INVENTOR(S) : Charlotte A. Kensil, et al

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 10, line 32, "40 nM" should read "40 mM".

Column 11, Table 2, last line, under the heading "HPLC Fraction" there should be a dash " - " to denote that no fraction was included as adjuvant with the antigen.

Column 12, line 1, "QA-17" should read "QA-7".

Column 20, line 49, "coolly" should read "commonly".

Column 21, line 32, "microliter" should read as "microtiter".

[redacted]

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,057,540

DATED : October 15, 1991

INVENTOR(S) : Charlotte A. Kensil, et al

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 21, lines 41 and 42, "AQ-7, AQ-17, AQ-18" should read "QA-7, QA-17, and QA-18".

Column 23, line 22, "monosuccharides" should read "monosaccharides".

Column 24, line 1, "mN" should read "mM".

Signed and Sealed this

First Day of June, 1993

Attest:

Michael K. Kirk

Attesting Officer

MICHAEL K. KIRK

Acting Commissioner of Patents and Trademarks

[redacted]

United States Patent [19]

Kensil et al.

[11] **Patent Number: 5,057,540**

[45] **Date of Patent: Oct. 15, 1991**

[54] SAPONIN ADJUVANT

[75] Inventors: **Charlotte A. Kensil**, Milford; **Dante J. Marciani**, Hopkinton, both of Mass.

[73] Assignee: **Cambridge Biotech Corporation**, Worcester, Mass.

[21] Appl. No.: **573,268**

[22] Filed: **Aug. 27, 1990**

Related U.S. Application Data

[63] Continuation of Ser. No. 200,754, May 31, 1988, abandoned, which is a continuation-in-part of Ser. No. 55,229, May 29, 1987, abandoned.

[51] **Int. Cl.**[5] **A61K 31/70**; A61K 31/705; A61K 39/00

[52] **U.S. Cl.** **514/25**; 514/26; 514/33; 514/35; 514/885; 424/88; 424/195.1; 536/4.1; 536/6.3; 536/5

[58] **Field of Search** 514/25, 26, 33, 35, 514/885; 424/88, 89, 195.1; 536/4.1, 6.3, 18.1, 127, 128, 5

[56] **References Cited**

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Primary Examiner—Ronald W. Griffin
Assistant Examiner—Nancy S. Carson
Attorney, Agent, or Firm—Sterne, Kessler, Goldstein & Fox

[57] **ABSTRACT**

Substantially pure saponins are disclosed. The saponins of the present invention are useful as immune adjuvants. Disclosed as well are immune response-provoking compositions comprising an antigen in admixture with the substantially pure saponins.

16 Claims, 23 Drawing Sheets

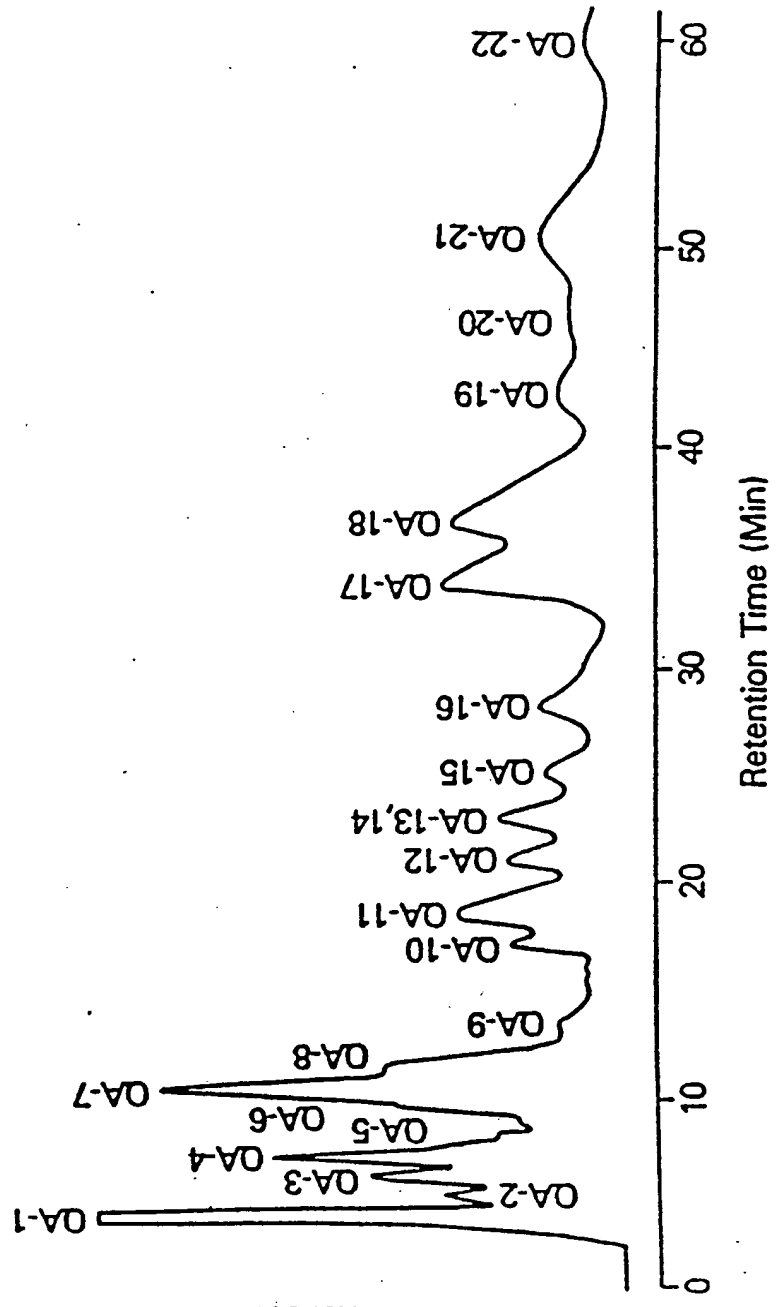
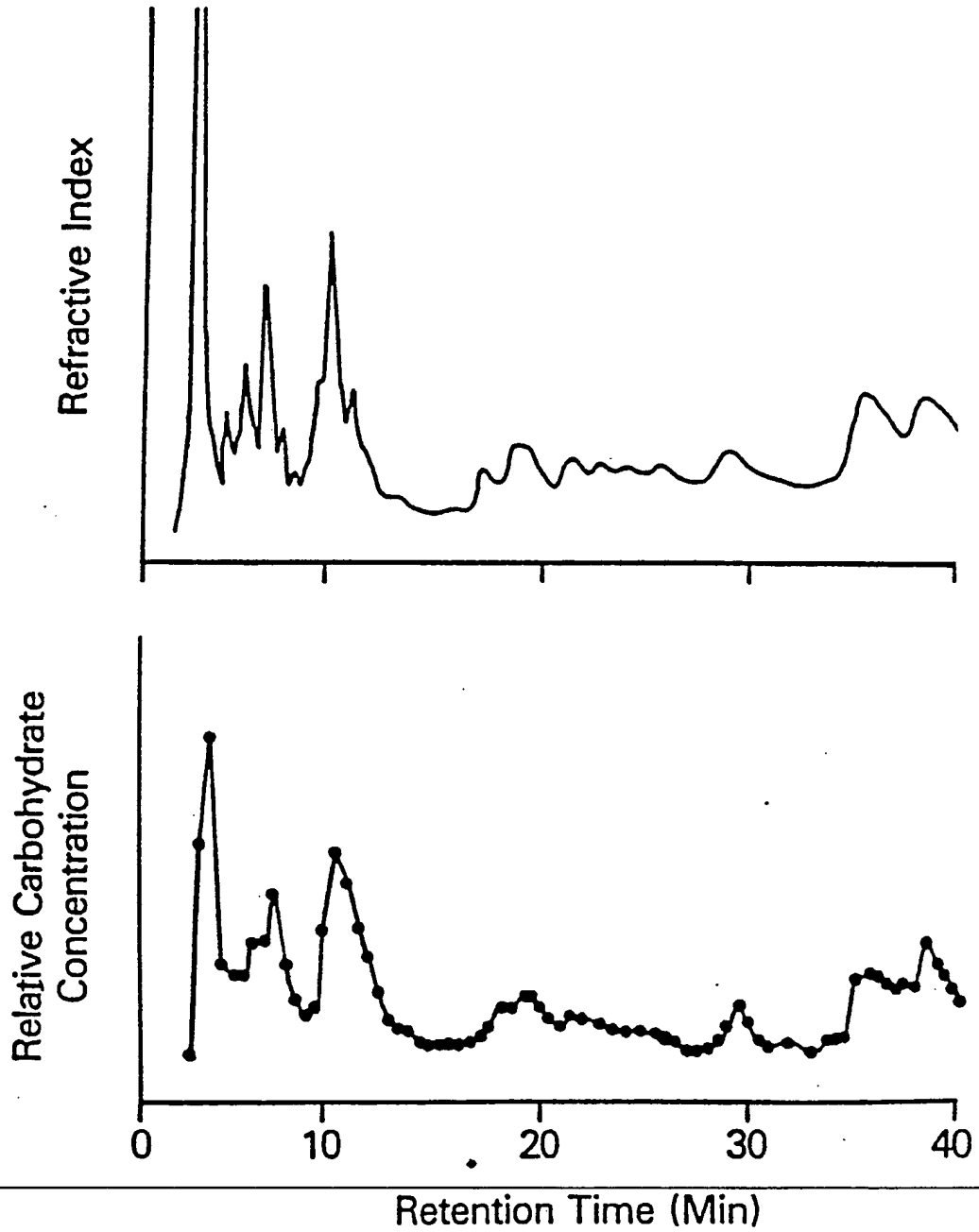

OA-1
OA-2
OA-3
OA-4
OA-5
OA-6
OA-7
OA-8
OA-9
OA-10
OA-11
OA-12
OA-13,14
OA-15
OA-16
OA-17
OA-18
OA-19
OA-20
OA-21
OA-22
Refractive Index
0
10
20
30
40
50
60
Retention Time (Min)

Figure 1

Figure 2


Refractive Index
Relative Carbohydrate Concentration
0
10
20
30
40
Retention Time (Min)

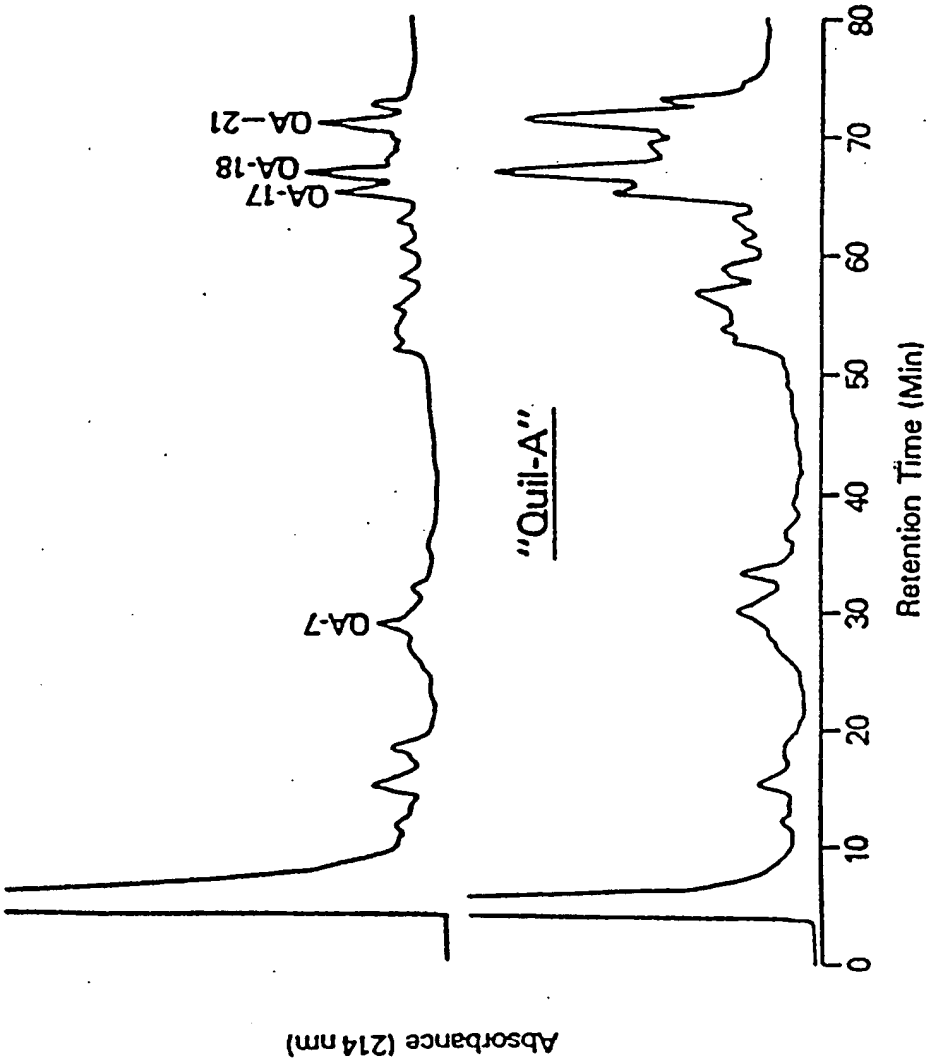
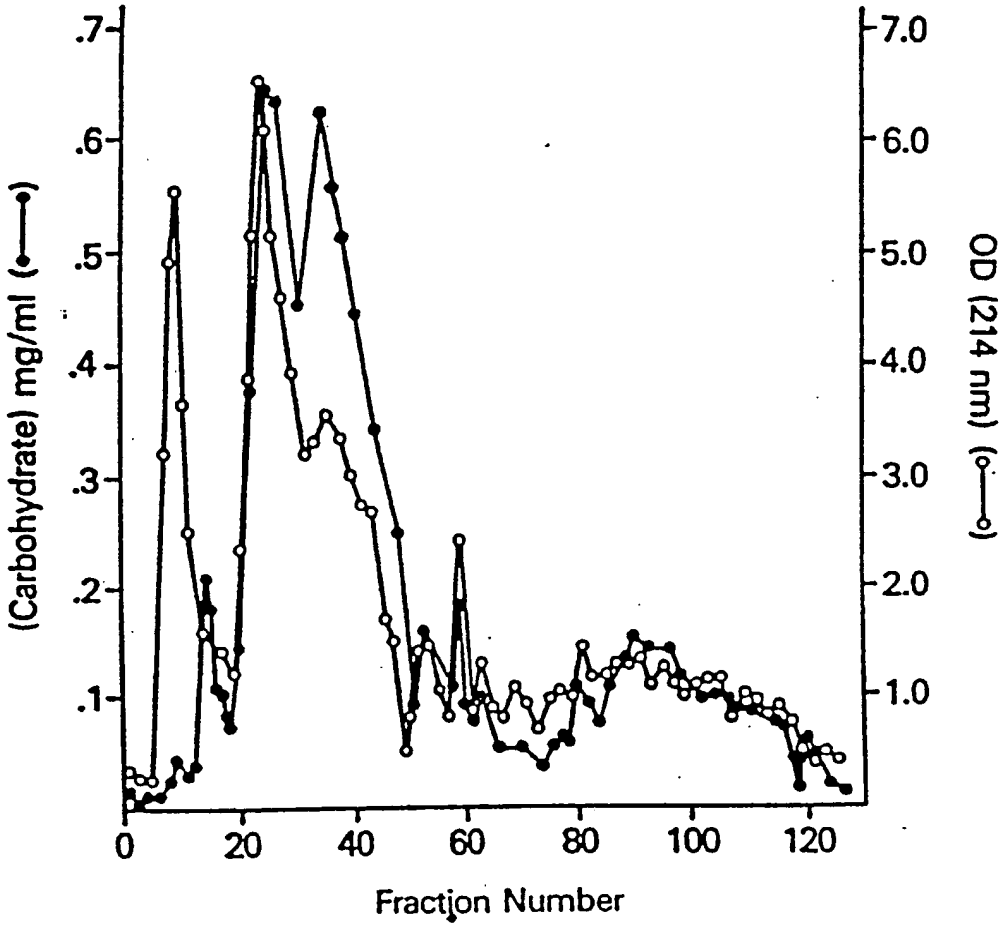

QA-21
QA-18
QA-17
QA-7
"Quil-A"
0
10
20
30
40
50
60
70
80
Retention Time (Min)
Absorbance (214 nm)

Figure 3

Figure 4A


.7
.6
.5
.4
.3
.2
.1
(Carbohydrate) mg/ml (●—●)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
OD (214 nm) (○—○)
0
20
40
60
80
100
120
Fraction Number

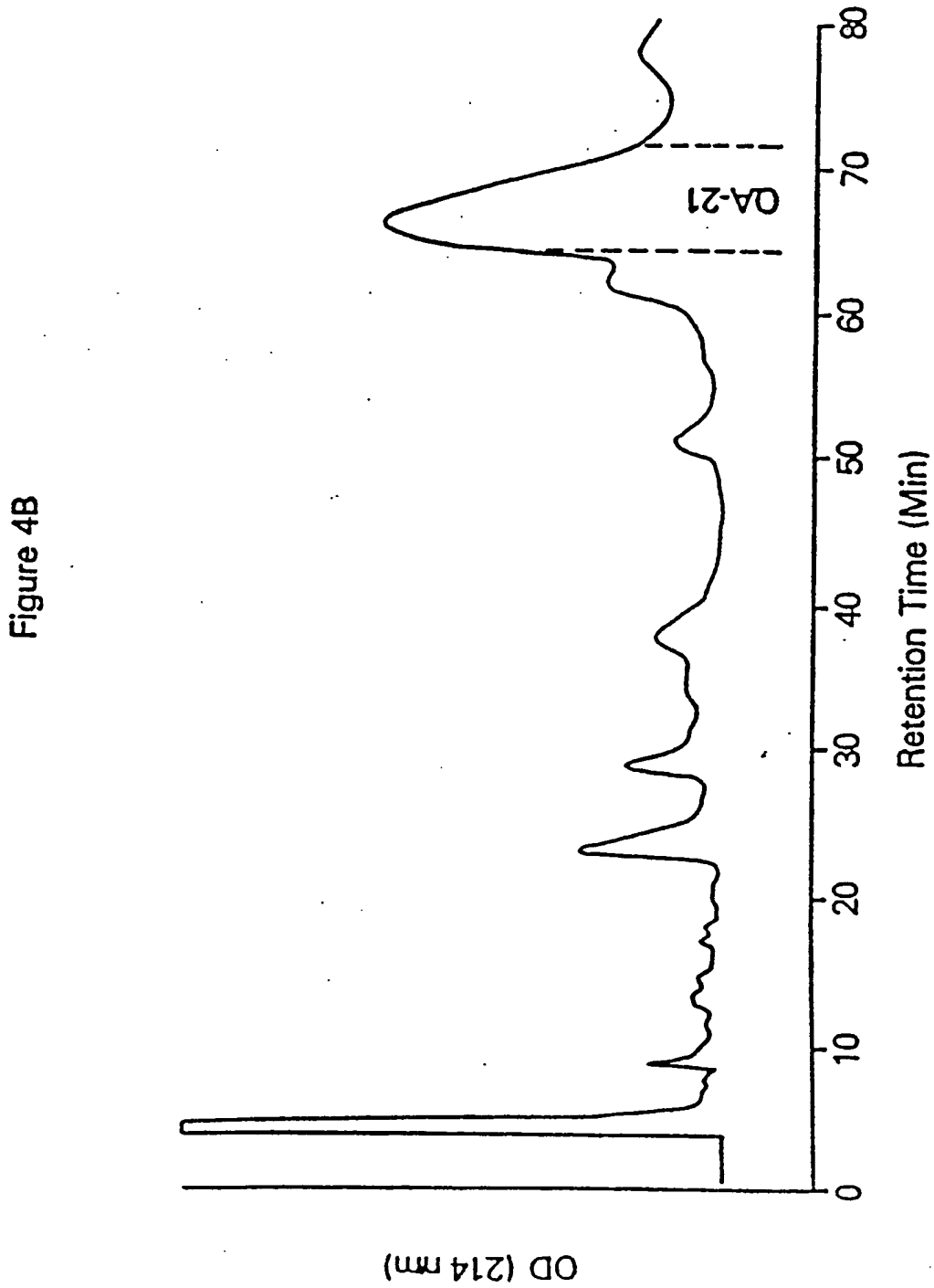


Figure 4B

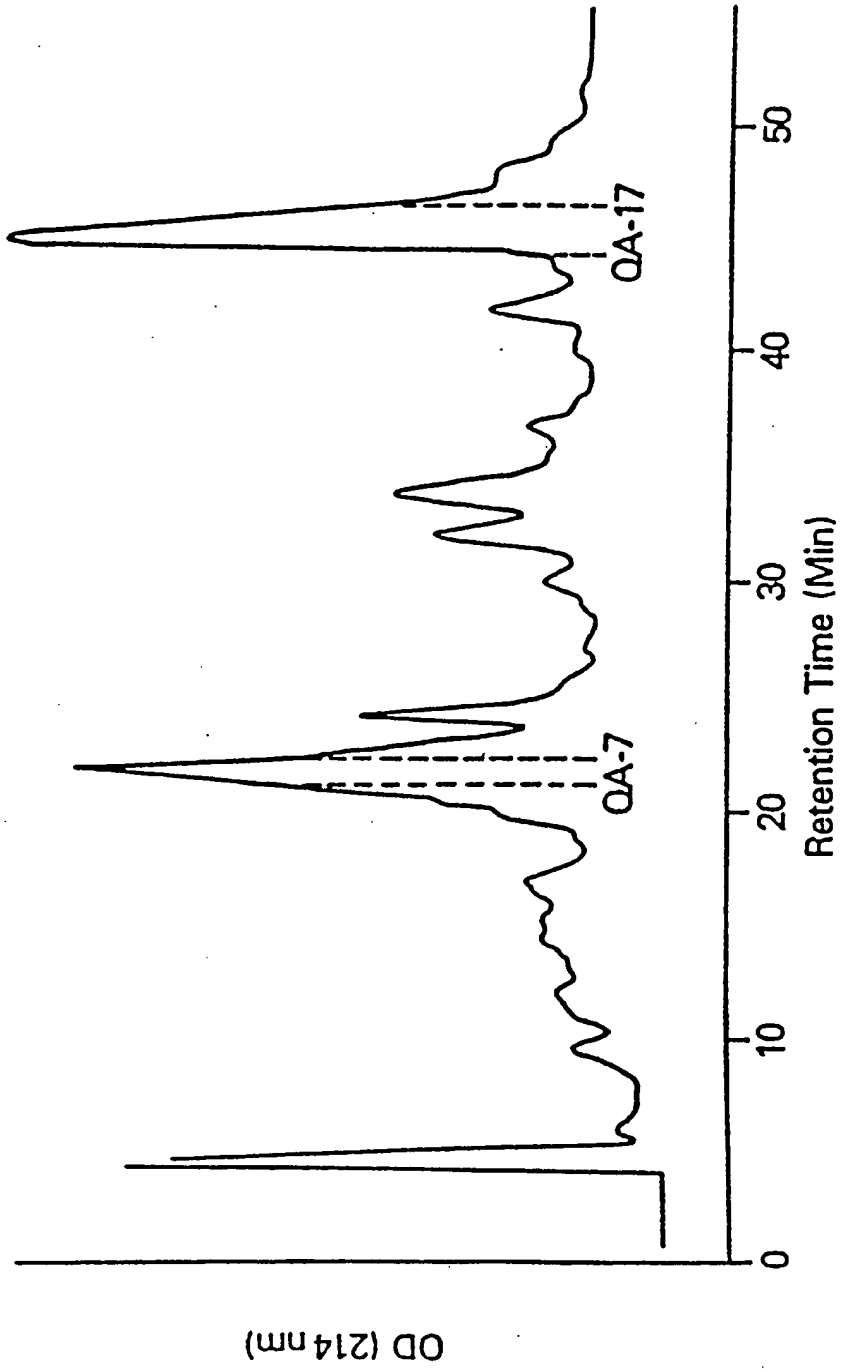


Figure 4D

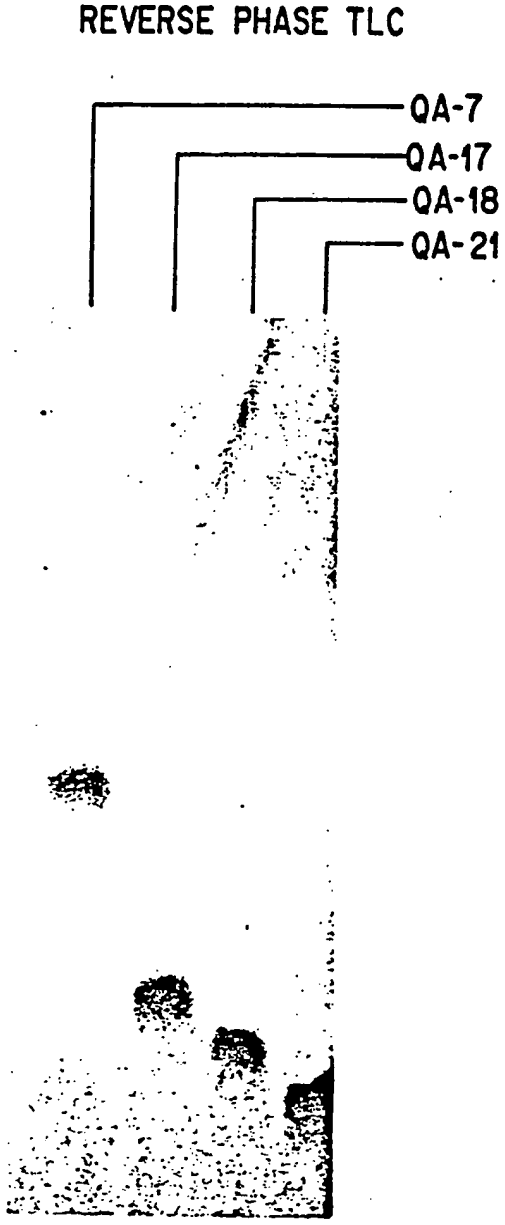


FIG. 5A

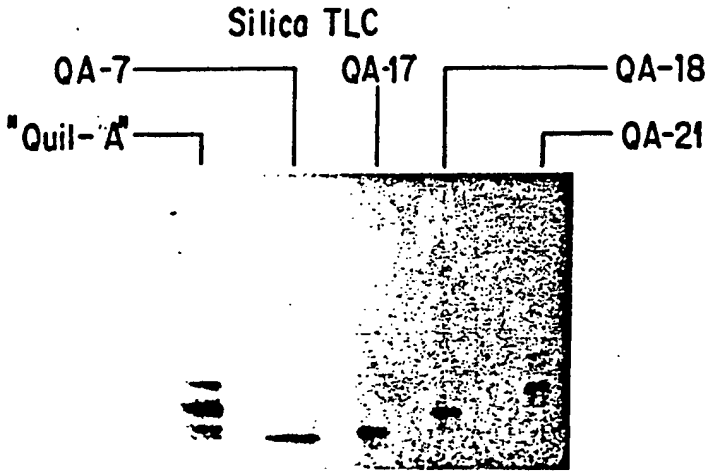
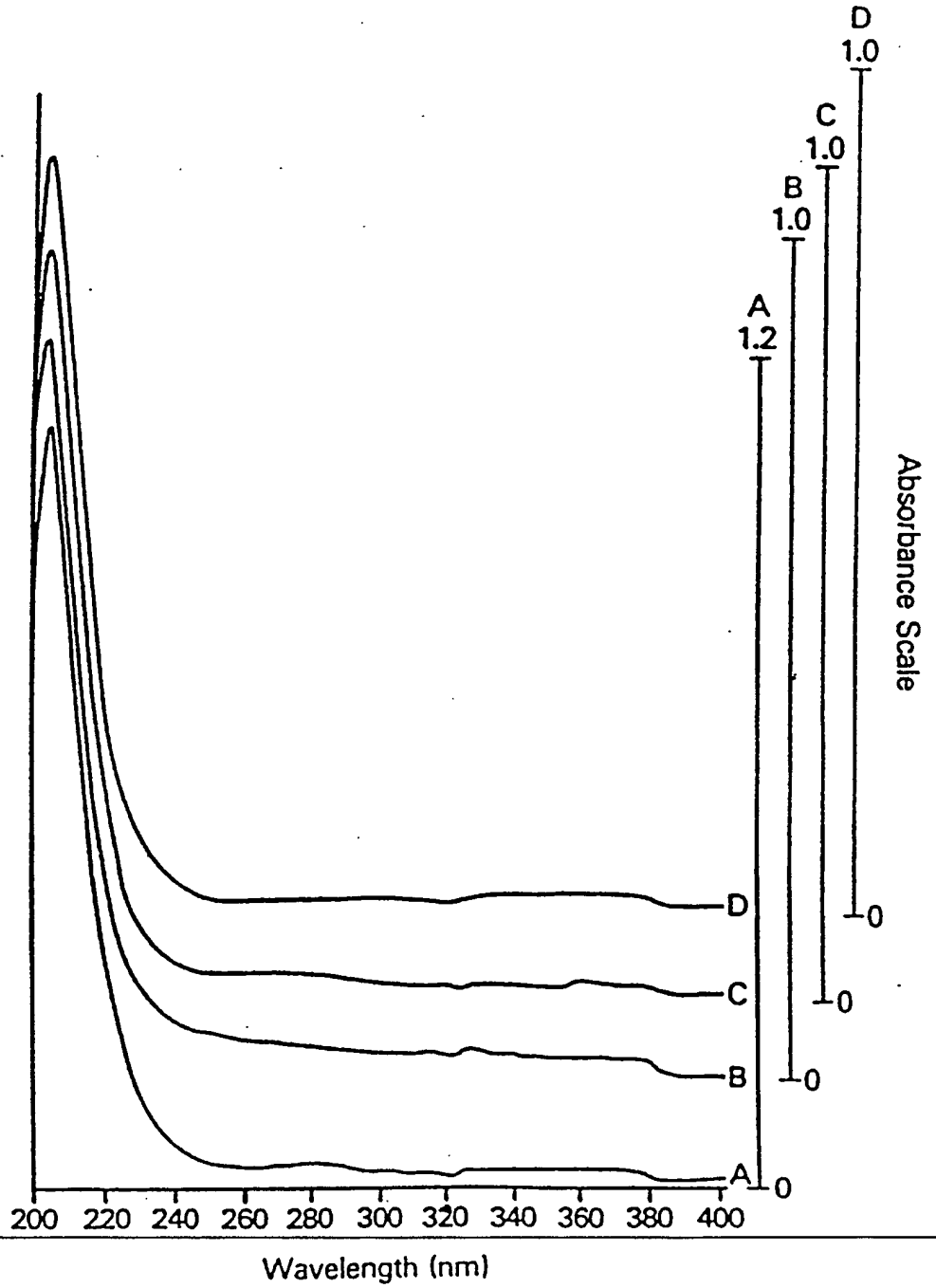


FIG. 5B

Figure 6


D
1.0
C
1.0
B
1.0
A
1.2
Absorbance Scale
D
C
B
A
0
0
0
0
200 220 240 260 280 300 320 340 360 380 400
Wavelength (nm)

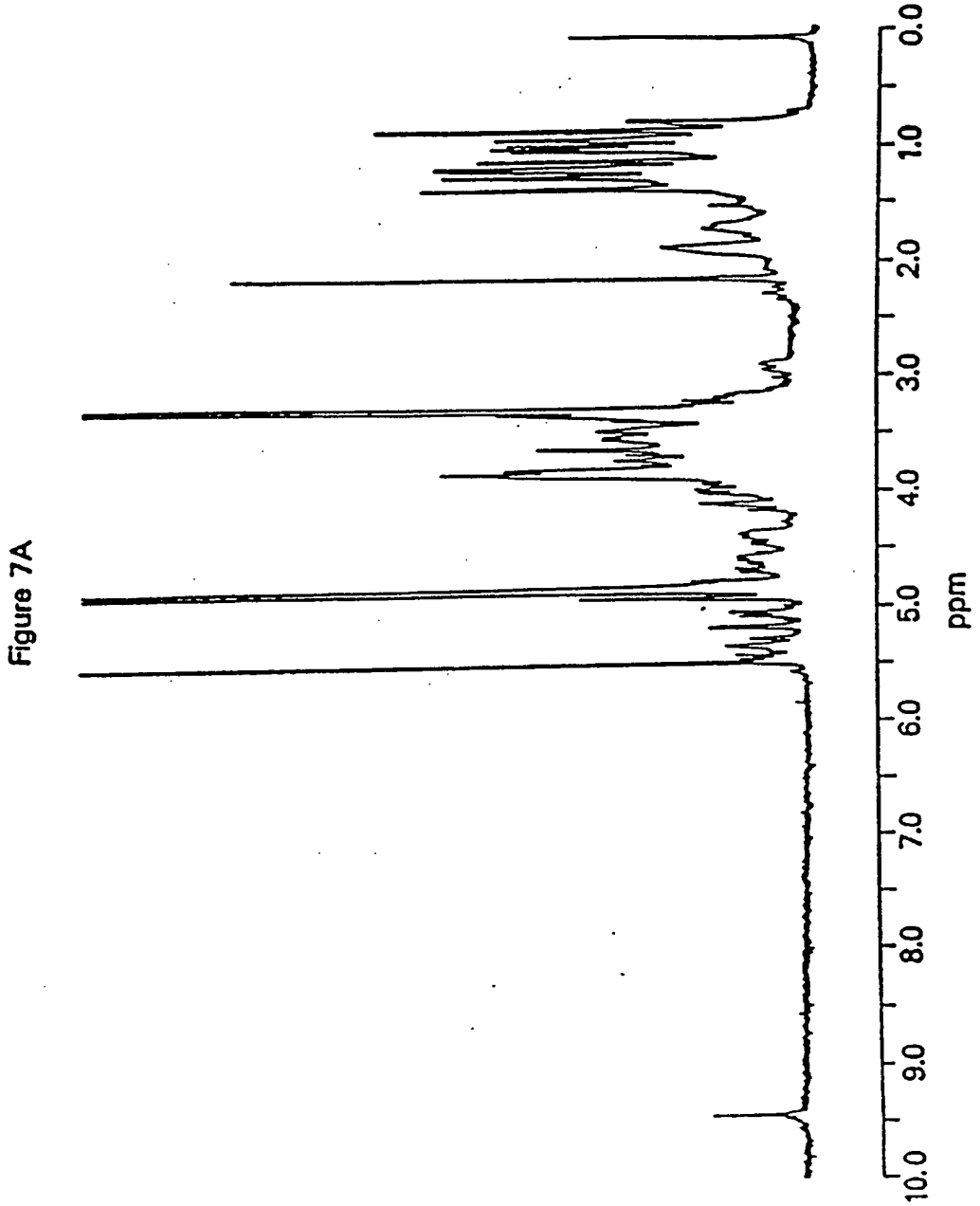


Figure 7A

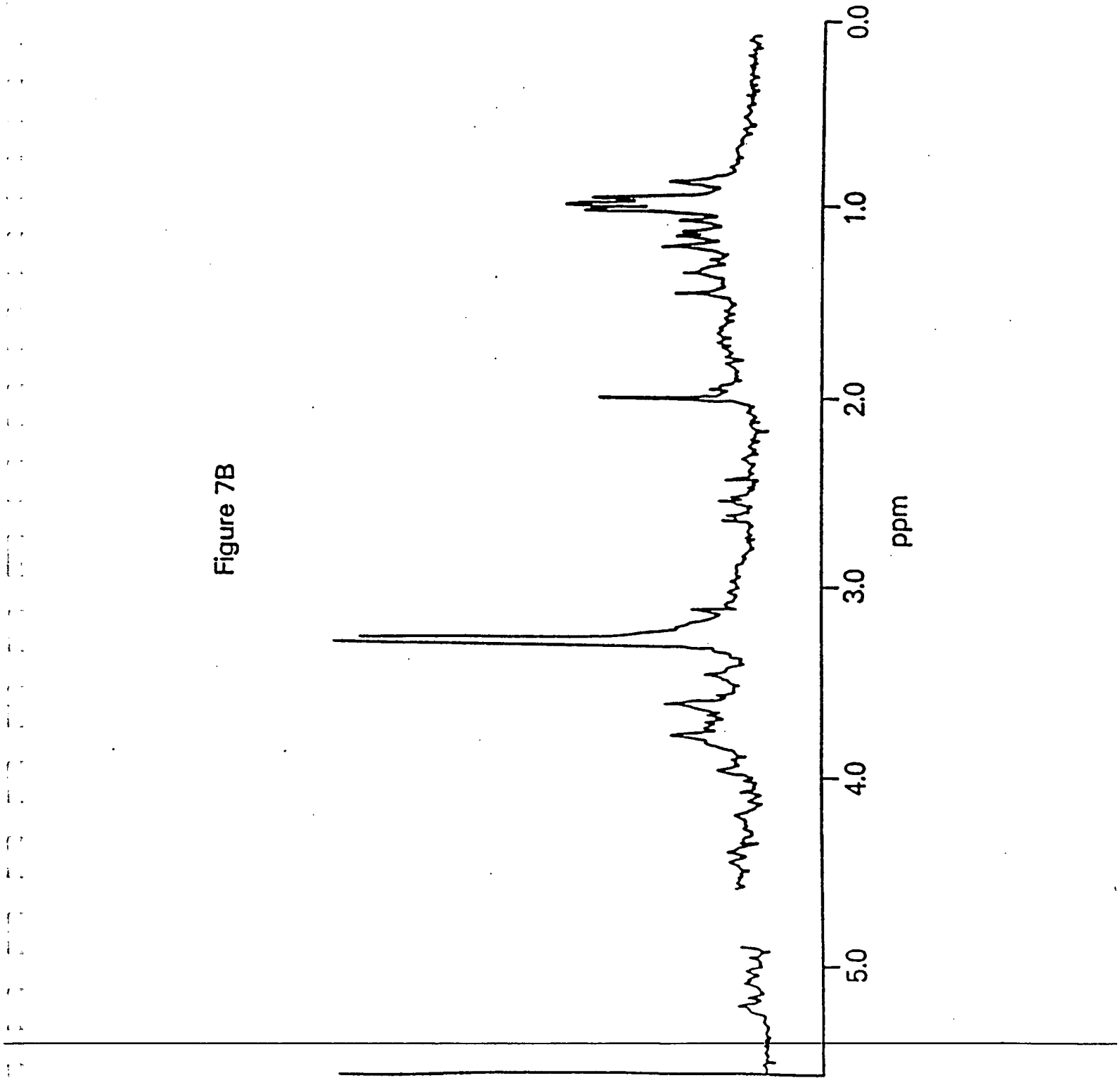


Figure 7B

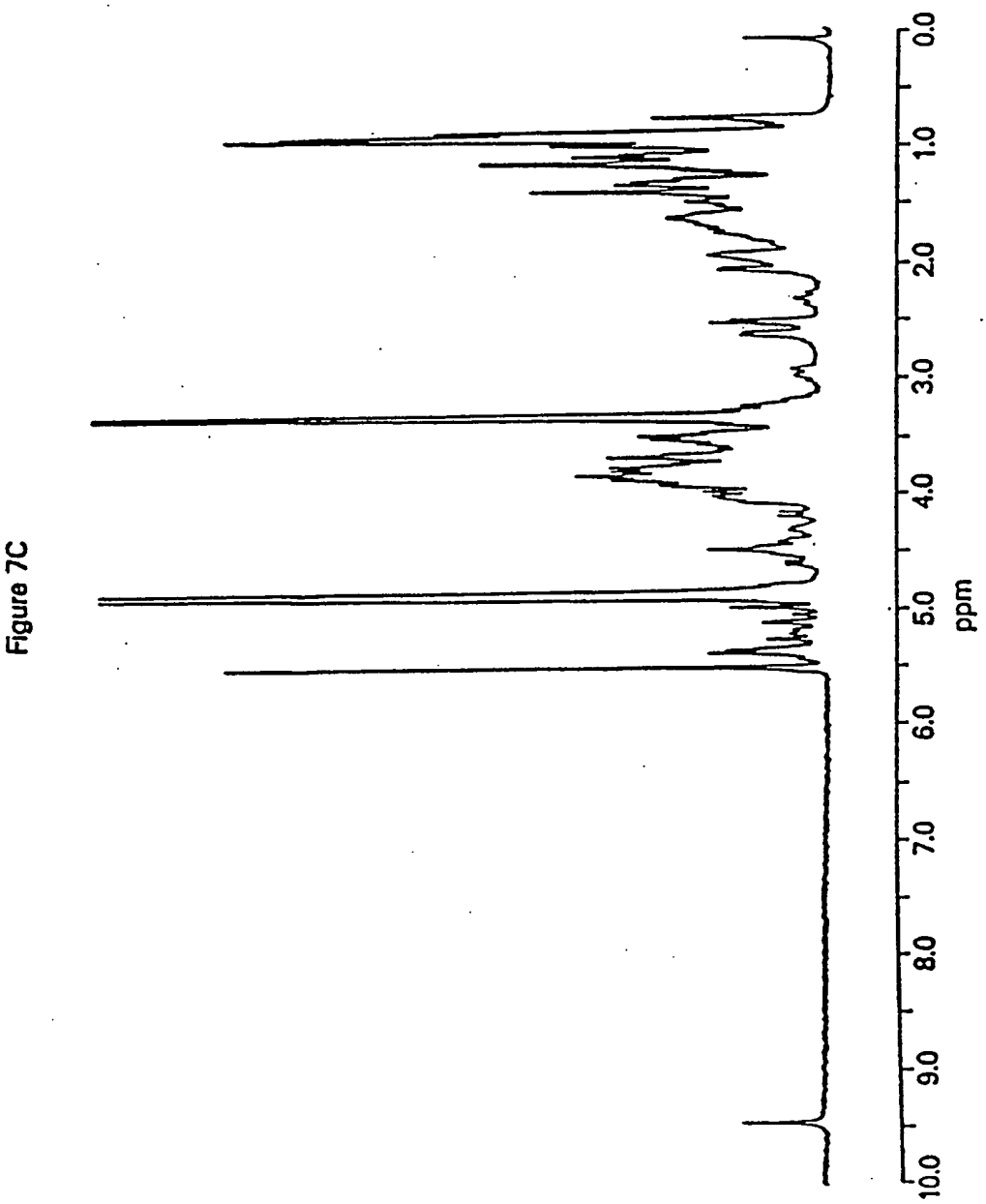


Figure 7C

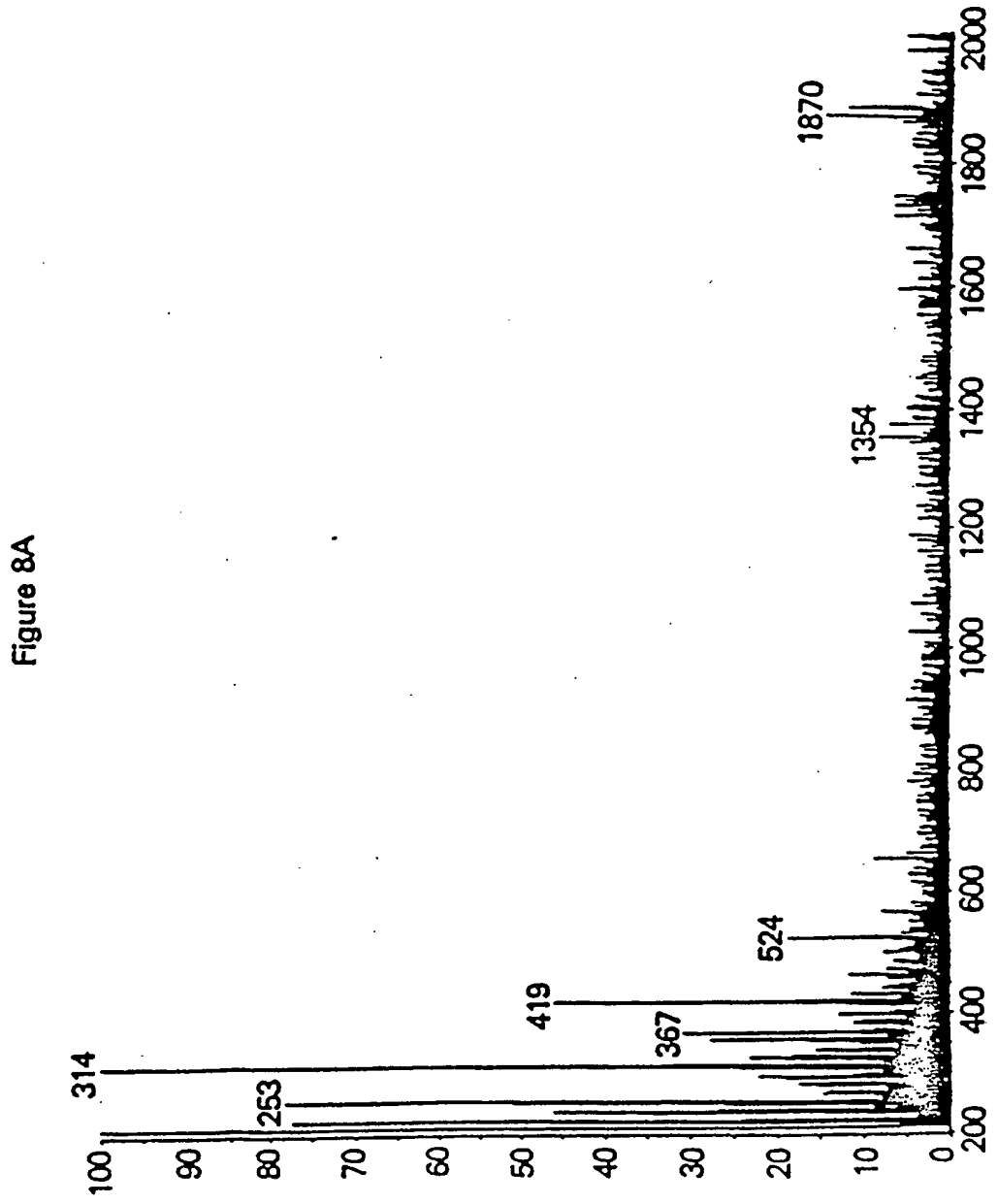

100
90
80
70
60
50
40
30
20
10
0
200
400
600
800
1000
1200
1400
1600
1800
2000
314
253
419
367
524
1354
1870

Figure 8A

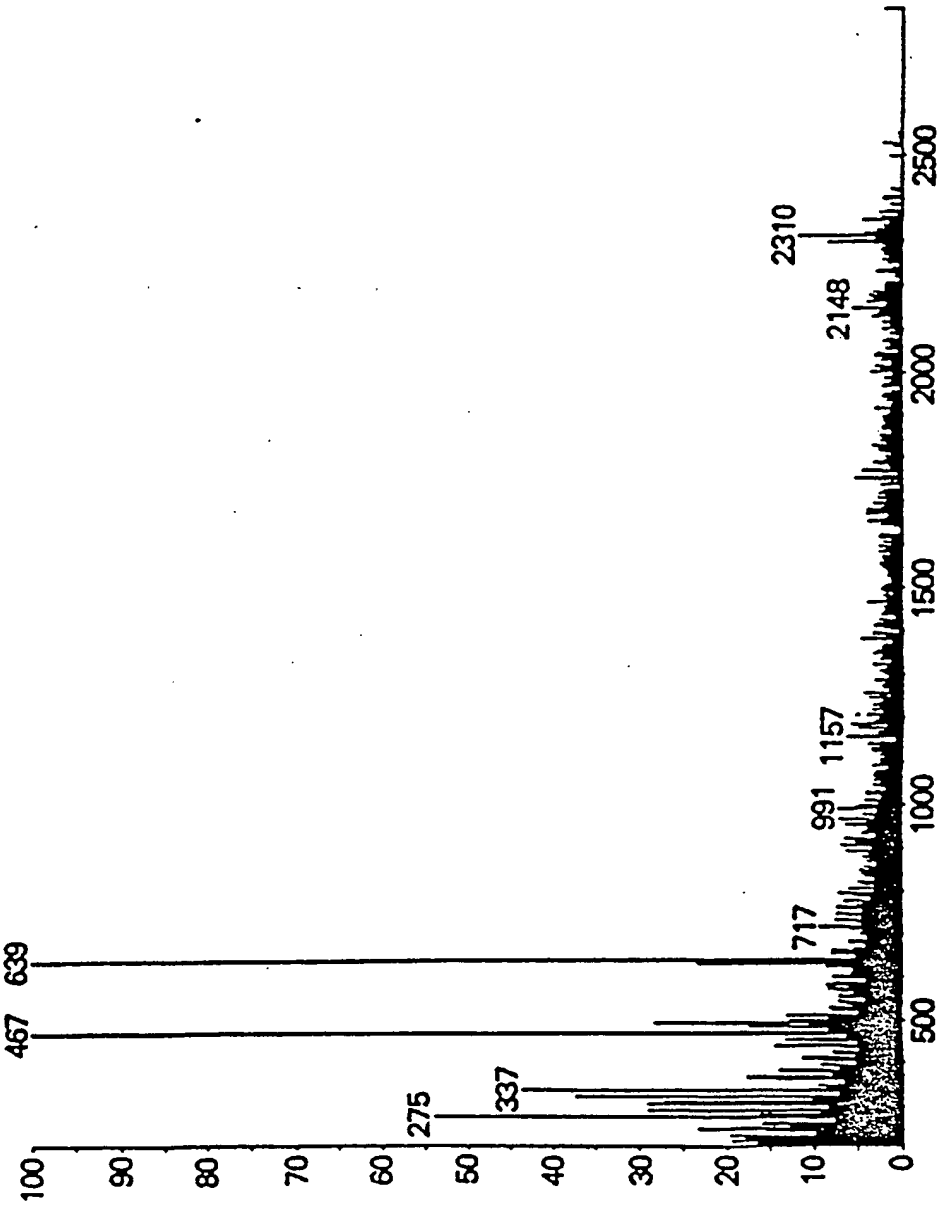

639
467
275
337
717
991
1157
2310
2148
100
90
80
70
60
50
40
30
20
10
0
500
1000
1500
2000
2500

Figure 8B

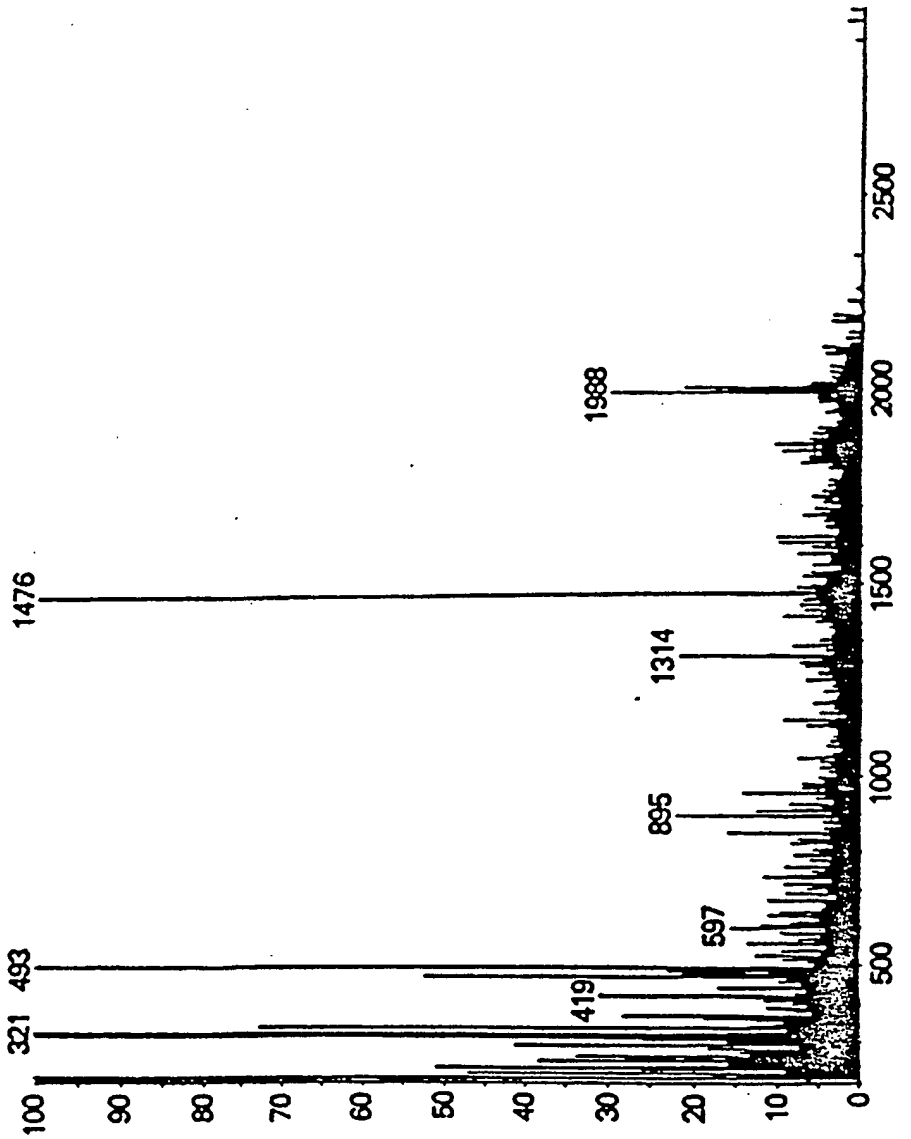

1988
1476
1314
895
597
493
419
321
2500
2000
1500
1000
500
100
90
80
70
60
50
40
30
20
10
0

Figure 8C

Figure 9

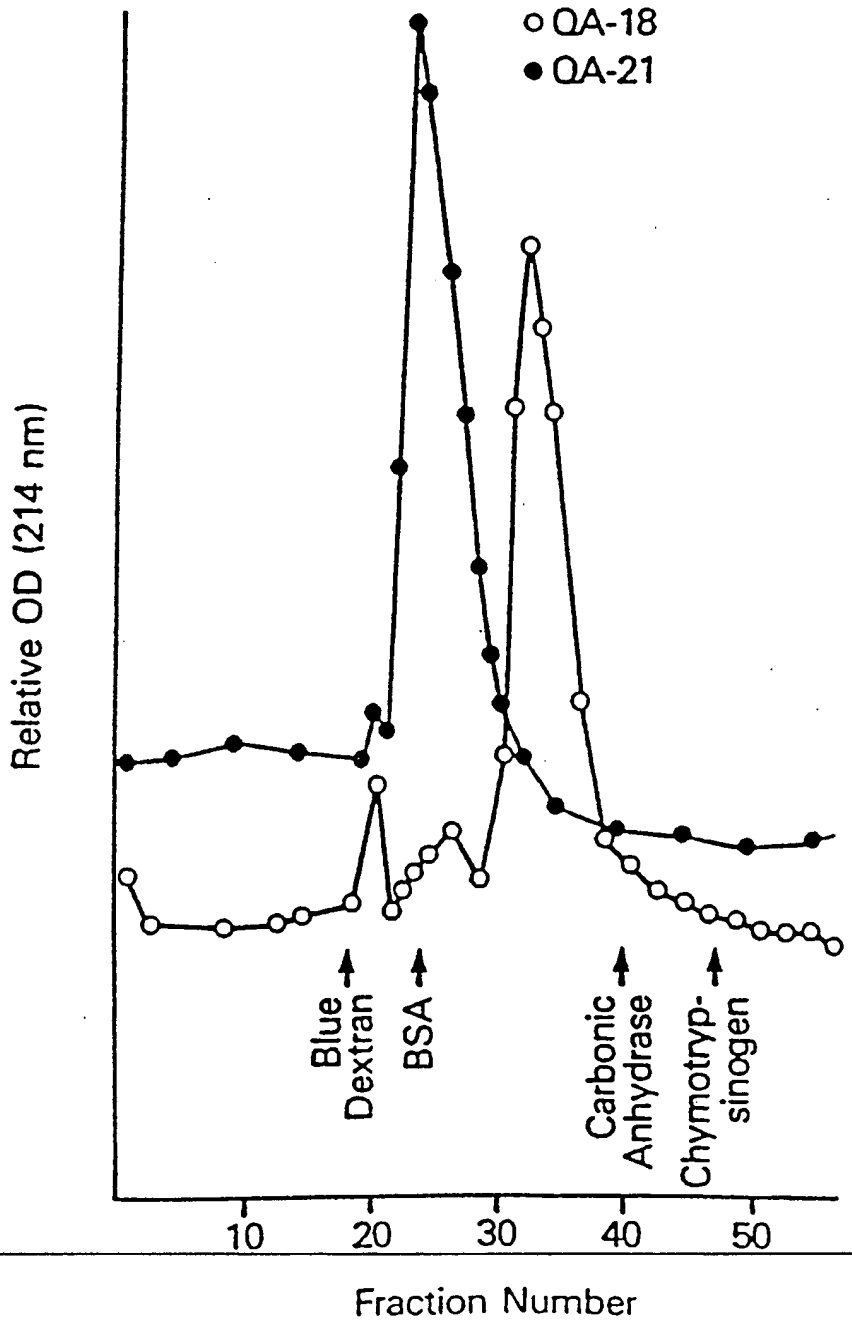

QA-18
QA-21
Relative OD (214 nm)
Blue Dextran
BSA
Carbonic Anhydrase
Chymotryp-sinogen
10
20
30
40
50
Fraction Number

Figure 10

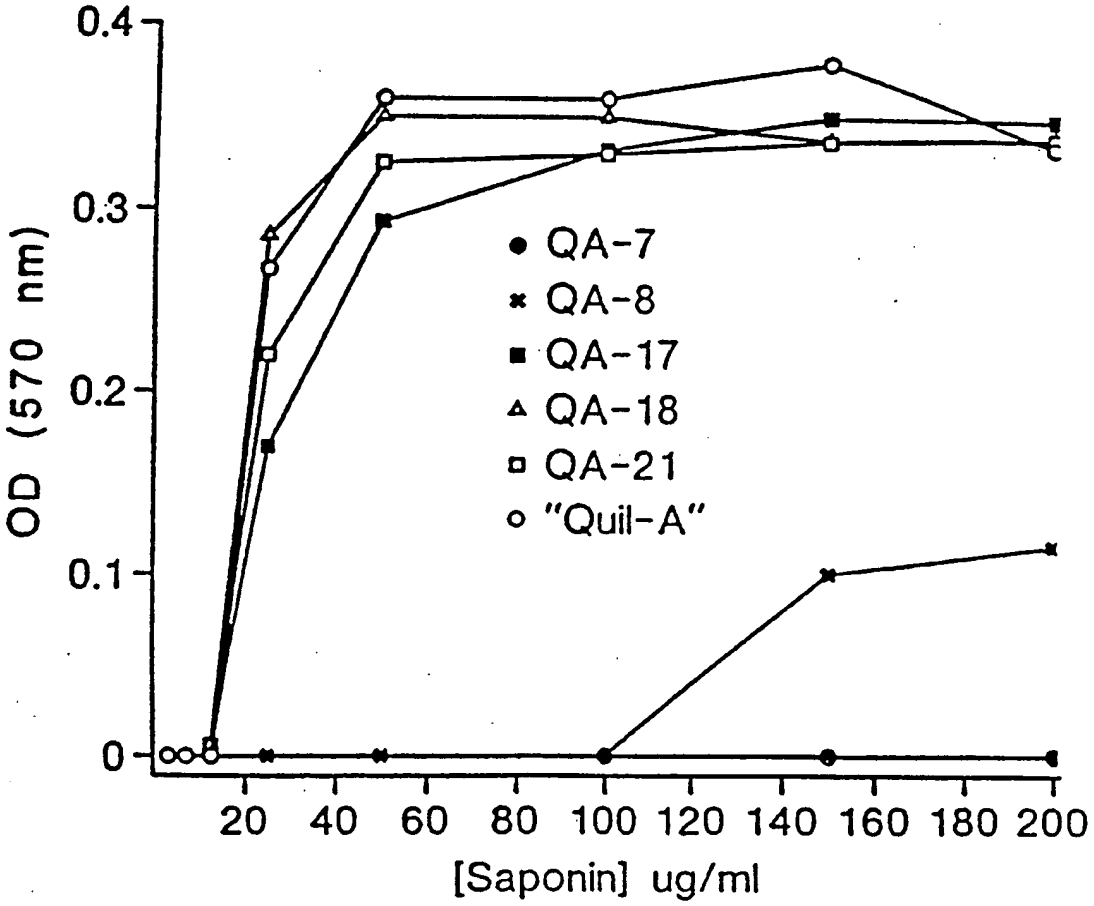
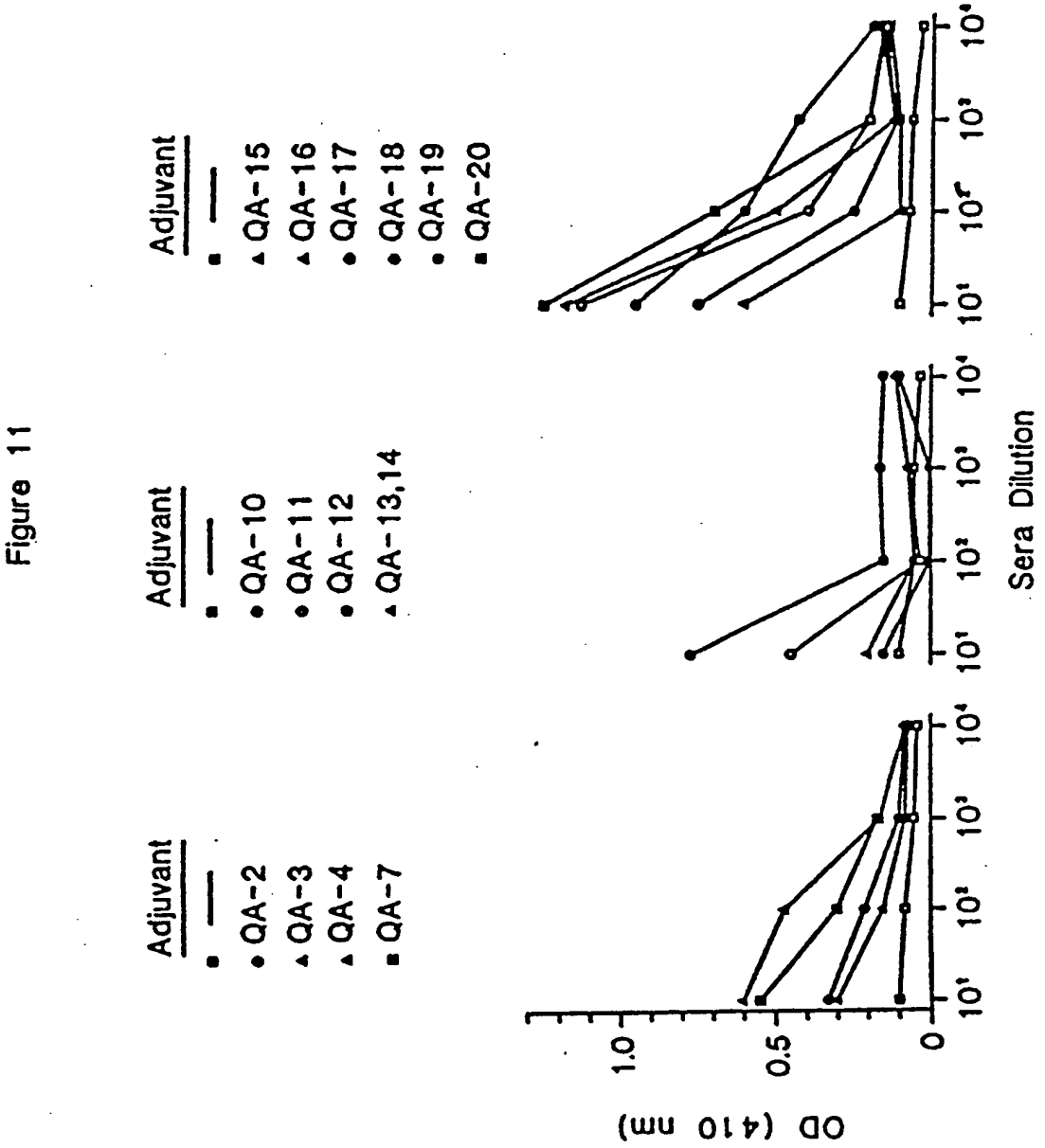

0.4
0.3
0.2
0.1
0
OD (570 nm)
QA-7
QA-8
QA-17
QA-18
QA-21
"Quil-A"
20 40 60 80 100 120 140 160 180 200
[Saponin] ug/ml

Figure 11


Adjuvant
QA-2
QA-3
QA-4
QA-7
Adjuvant
QA-10
QA-11
QA-12
QA-13,14
Adjuvant
QA-15
QA-16
QA-17
QA-18
QA-19
QA-20
OD (410 nm)
0
0.5
1.0
Sera Dilution
10¹
10²
10³
10⁴

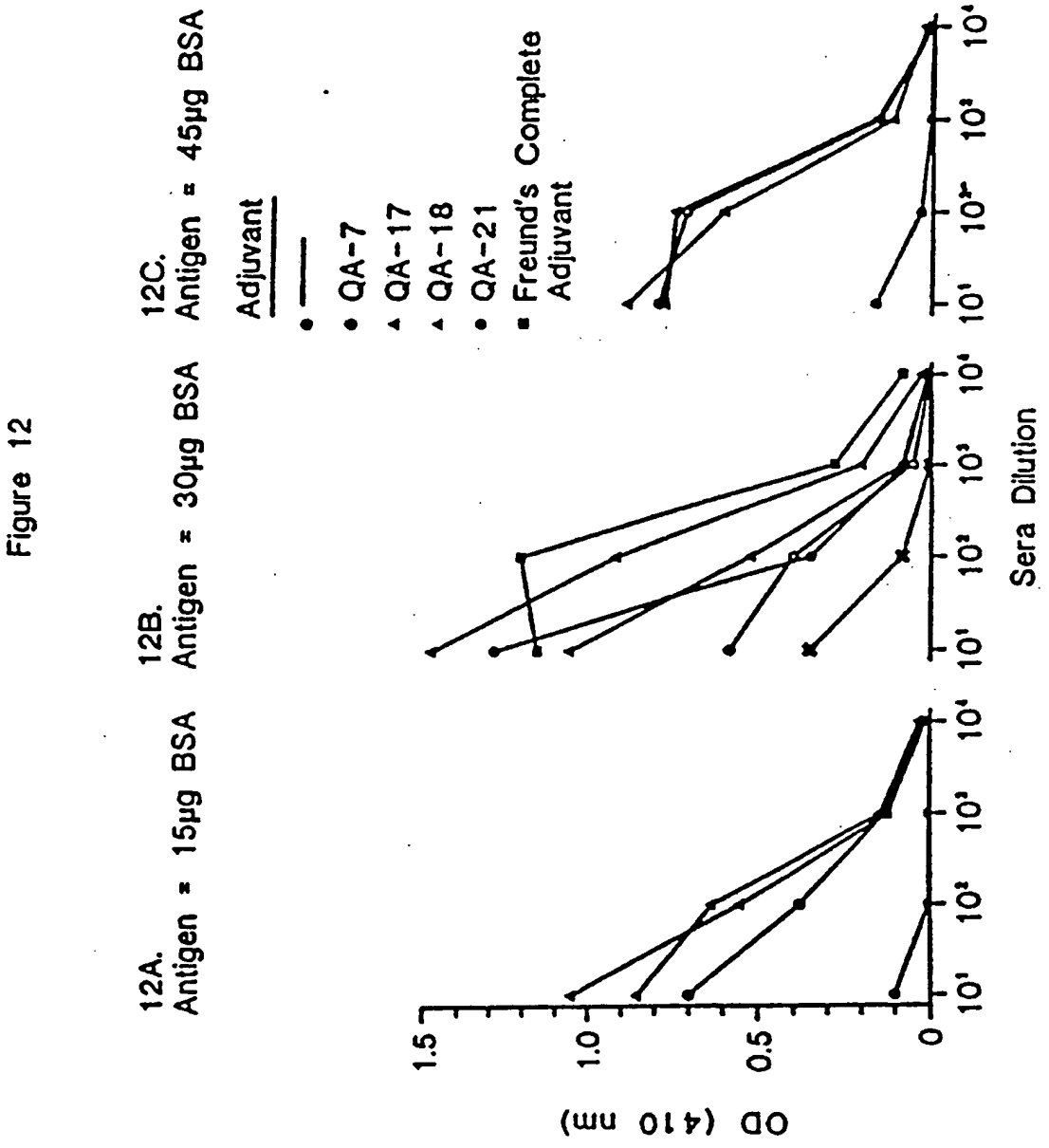

12A.
Antigen = 15µg BSA
12B.
Antigen = 30µg BSA
12C.
Antigen = 45µg BSA
Adjuvant
—
QA-7
QA-17
QA-18
QA-21
Freund's Complete Adjuvant
OD (410 nm)
1.5
1.0
0.5
0
10¹
10²
10³
10⁴
Sera Dilution

Figure 12

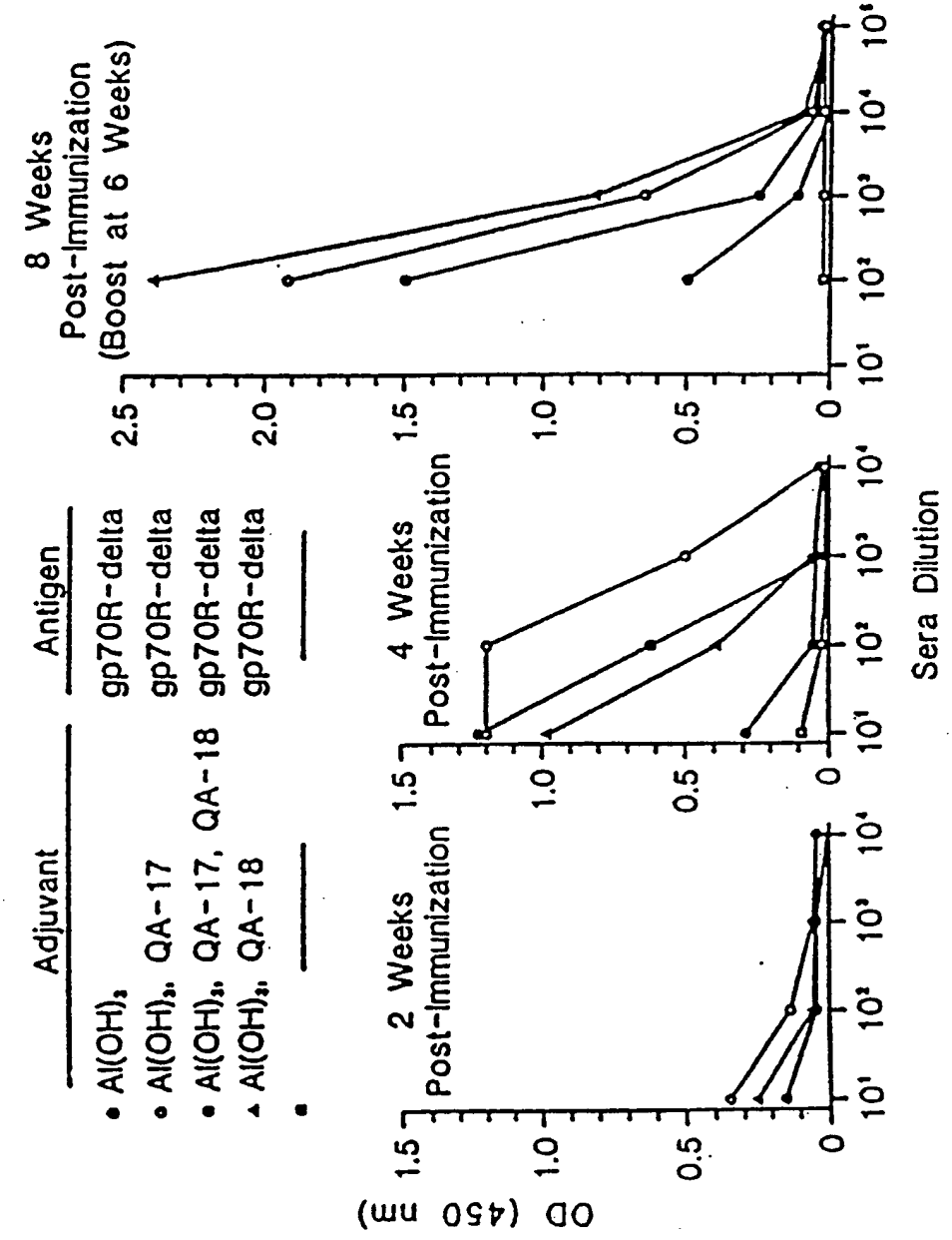

Adjuvant
Antigen
• Al(OH)3 gp70R-delta
○ Al(OH)3, QA-17 gp70R-delta
▪ Al(OH)3, QA-17, QA-18 gp70R-delta
▲ Al(OH)3, QA-18 gp70R-delta
▪ ———
2 Weeks Post-Immunization
4 Weeks Post-Immunization
8 Weeks Post-Immunization (Boost at 6 Weeks)
OD (450 nm)
Sera Dilution

Figure 13

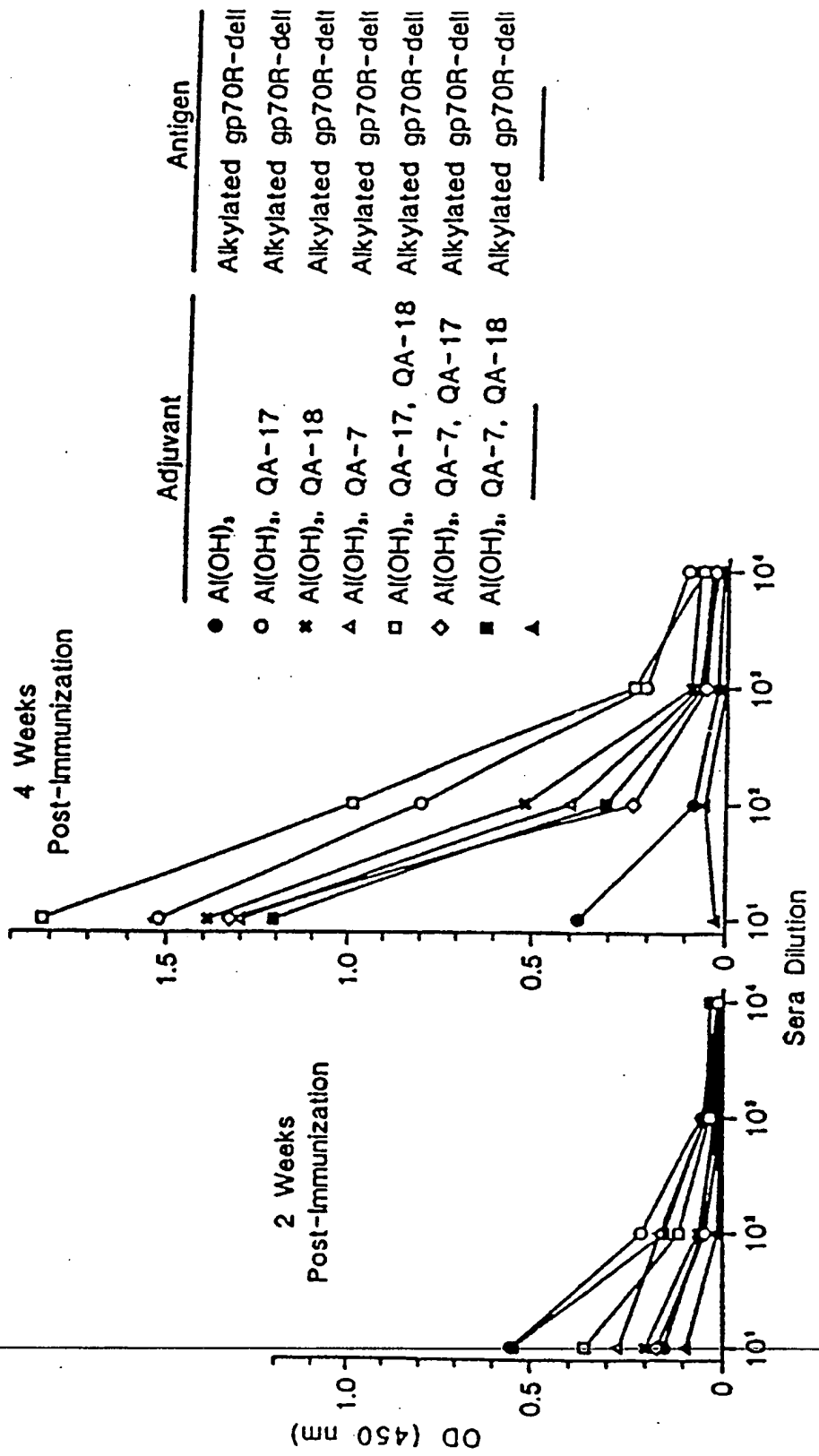
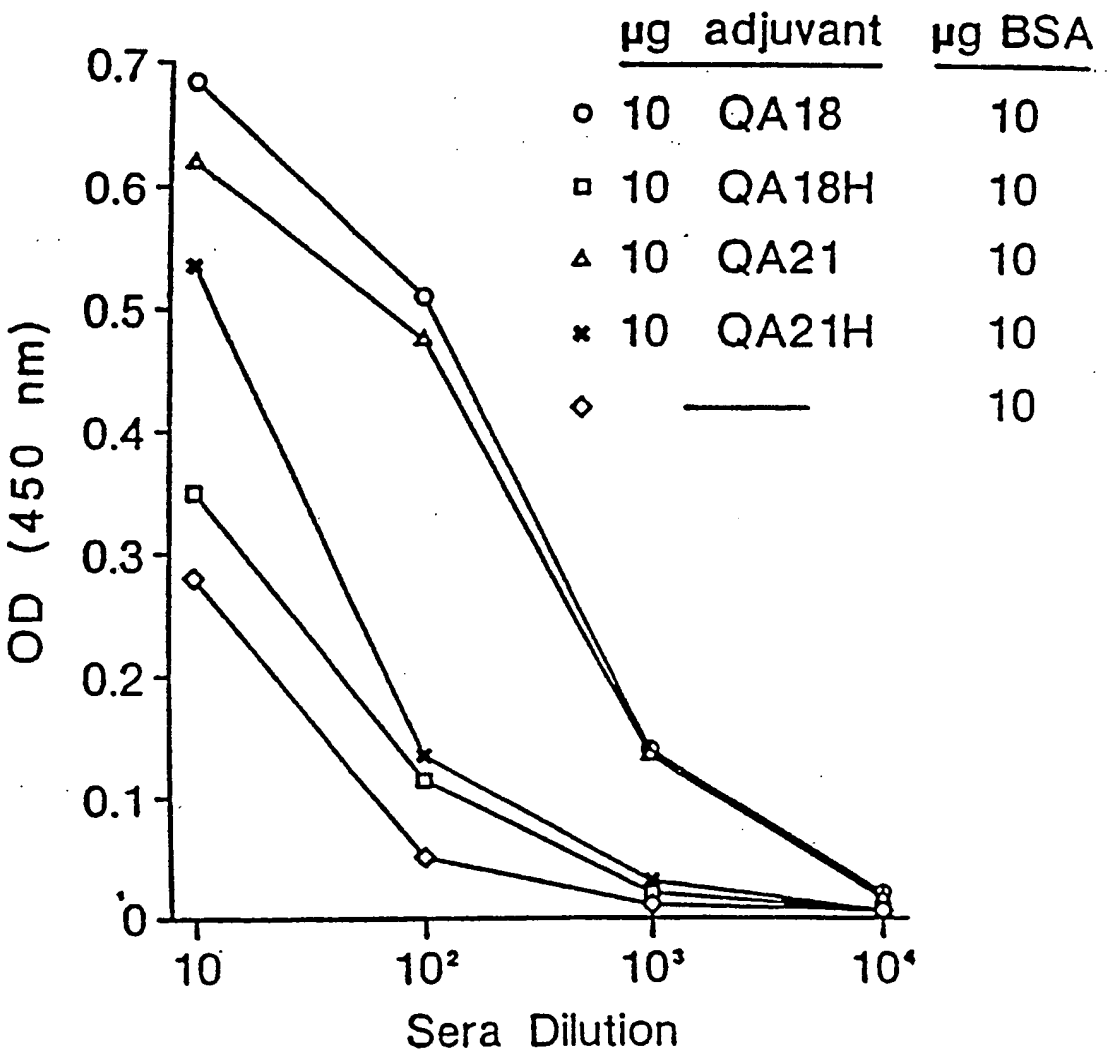

Adjuvant
Antigen
Al(OH)3
Alkylated gp70R-del
Al(OH)3, QA-17
Alkylated gp70R-del
Al(OH)3, QA-18
Alkylated gp70R-del
Al(OH)3, QA-7
Alkylated gp70R-del
Al(OH)3, QA-17, QA-18
Alkylated gp70R-del
Al(OH)3, QA-7, QA-17
Alkylated gp70R-del
Al(OH)3, QA-7, QA-18
Alkylated gp70R-del
4 Weeks Post-Immunization
2 Weeks Post-Immunization
OD (450 nm)
Sera Dilution
0
0.5
1.0
1.5
10¹
10²
10³
10⁴

Figure 14

Figure 15


μg adjuvant
μg BSA
10 QA18 10
10 QA18H 10
10 QA21 10
10 QA21H 10
10
OD (450 nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10
10²
10³
10⁴
Sera Dilution

SAPONIN ADJUVANT

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation of application Ser. No. 07/200,754, filed 05/31/88 which is a continuation-in-part of U.S. patent application Ser. No. 055,229 filed May 29, 1987 and having the title "Saponin Adjuvant" both now abandoned.

This application is also related to U.S. patent application Ser. No. 55,298, which is a continuation-in-part of U.S. patent application Ser. No. 868,585, entitled "Method of Preparation and Use For Feline Leukemia Virus Antigens," in the names of Beltz et al.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to the field of immune adjuvants, the process for production thereof, and the use thereof as immune adjuvants and vaccines.

2. Brief Description of the Background Art

Quillaja saponins are a mixture of triterpene glycosides extracted from the bark of the tree *Quillaja saponaria*. Crude saponins have been extensively employed as adjuvants in vaccines against foot and mouth disease, and in amplifying the protective immunity conferred by experimental vaccines against protozoal parasites such as *Trypanosoma cruzi* plasmodium and also the humoral response to sheep red blood cells (SRBC). (Bomford, *Int. Arch. Allerg. appl. Immun.*, 67:127 (1982)).

Saponins are natural products which have been characterized by a number of common properties. The ability to produce foam in aqueous solution gave the name to the group. Further characteristics are the hemolytic activity, the toxicity for fish, the complexing with cholesterol, and in some cases antibiotic activity. Kofler, *Die Saponine* (Springer Berlag), Berlin, 1927; Tschesche et al., *Chemine und Biologic der Saponine. Fortscher. Chem. Oro. Naturst.* XXX:461 (1972).

The common properties of saponins are not reflected in a common chemical composition. Although all saponins are glycosides, the aglycone may belong to the steroids, the triterpenoids, or the steroidalcaloids. The number of sugar and sugar chains attached to the glycosidic bonds may vary greatly. Saponins have been produced commercially and have many uses. The commercially available Quillaja saponins are crude mixtures which, because of their variability, are not desirable for use in veterinary practice or in pharmaceutical compositions for man. Because of the variability and heterogeneity, each batch must be tested in animal experiments to determine adjuvant activity and toxicity. The impurities in the commercially available products may produce adverse reactions. In addition, the content of the active substance in a given batch of saponin may vary, thereby decreasing the reproducibility from batch to batch.

An early attempt to purify Quillaja saponin adjuvants was made by Dalsgaard, *Archiv fuer die gesamte Virusforschung* 44:243 (1974). Dalsgaard partially purified an aqueous extract of the saponin adjuvant material from *Quillaja saponaria Molina*. Dalsgaard's preparation, commercially available from Superfos under the name "Quil-A," has been isolated from the bark of the South American tree, *Quillaja saponaria Molina*, and is characterized chemically as a carbohydrate moiety in glycosidic linkage to the triterpenoid quillaic acid. However,

while the saponin Quil A of Dalsgaard presents a definite improvement over the previously available commercial saponins, it also shows considerable heterogeneity.

Higuchi et al., *Phytochemistry* 26:229 (January, 1987) treated a crude Quillaja saponin mixture with alkaline hydrolysis in 6% NH_4HCO_3 in 50% methanol and generated two major desacylsaponins, termed DS-1 and DS-2. DS-1 was shown to contain glucuronic acid, galactose, xylose, fucose, rhamnose, apiose, and Quillajic acid, whereas DS-2 contained these same components plus an additional glucose. Byproducts of this deacylation produced multiple components including 3,5-dihydroxy-6-methyloctanoic acid, 3,5-dihydroxy-6-methyloctanic acid, 5-α-L-arabinofuranoside and 5-O-α-L-rhamnopyranosyl-(1->2)-α-L-arabinofuranoside (Higuchi et al., *Phytochemistry* 26:2357 (August, 1987).

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 shows the refractive index profile of dialyzed, methanol-solubilized Quillaja bark extract on reverse phase-HPLC.

FIG. 2 shows that the refractive index peaks of the above sample correspond to carbohydrate peaks.

FIG. 3 shows the comparison of Superfos "Quil-A" and dialyzed methanol soluble bark extract by HPLC.

FIG. 4 shows the purification of QA-7, QA-17, QA-18, QA-19, and QA-21 from "Quil-A," a crude saponin mixture, by silica chromatography (4A) and subsequent reverse phase chromatography (4B, 4C, 4D).

FIG. 5 demonstrates the purity of QA-7, QA-17, QA-18, and QA-21 by reverse phase (5A) and normal phase (5B) thin layer chromatography.

FIG. 6A shows the UV spectrum of QA-7. FIG. 6B shows the UV spectrum of QA-17. FIG. 6C shows the UV spectrum of QA-18. FIG. 6D shows the UV spectrum of QA-21.

FIG. 7A shows 'H Nuclear Magnetic Resonance ("NMR") of QA-7. FIG. 7B shows 'H NMR of QA-18. FIG. 7C shows 'H NMR of QA-21.

FIG. 8A shows the mass spectroscopy-fast atom bombardment ("MS-FAB") spectrum of QA-7. FIG. 8B shows the MS-FAB spectrum of QA-17. FIG. 8C shows the MS-FAB spectrum of QA-21.

FIG. 9 shows the elution profile of pure QA-18 micelles and pure QA-21 micelles by gel filtration on Bio-Gel P-200 in PBS equilibrated with the critical micellar concentration of the same saponin and a comparison with the elution position of standard proteins.

FIG. 10 shows the hemolysis of sheep red blood cells by QA-7, QA-8, QA-17, QA-18, QA-21, and Superfos "Quil-A."

FIG. 11 shows the typical endpoint titers for immunization with BSA antigen in the presence of HPLC-purified fractions of bark extract. Absorbance due to antigen-specific antibody binding was plotted as a function of the logarithm of the sera dilution.

FIG. 12 demonstrates the comparison of the adjuvant effects of QA-7, QA-17, QA-18 and QA-21 at various antigen concentrations and with Freund's complete adjuvant on immunization with the antigen BSA.

FIG. 13 shows the adjuvant effects of HPLC-purified adjuvants used in conjunction with $Al(OH)_3$, another adjuvant, on the immunization with the antigen gp70R-delta.

FIG. 14 summarizes the effects of HPLC-purified Quillaja saponins alone and in combination with each

other and with another adjuvant on the immunization with the antigen alkylated gp70R-delta.

FIG. 15 shows a comparison of the adjuvant effects of QA-18, QA-18H, QA-21, and QA-21H on immunization with the antigen BSA.

SUMMARY OF THE INVENTION

A need exists for a substantially pure saponin that can be used as an adjuvant in relatively low quantities with low toxicity and side effects. Accordingly, the present invention provides substantially pure saponin adjuvants, the method for the purification thereof and a method for the use of the substantially pure saponins as immune adjuvants. The invention further includes immune response-provoking compositions comprising the saponin adjuvants in combination with an antigen component.

Adjuvant saponins have been identified and purified from an aqueous extract of the bark of the South American tree, *Quillaja saponaria Molina.* At least 22 peaks with saponin activity were separable. The predominant purified Quillaja saponins have been identified as QA-7, QA-17, QA-18, and QA-21. These saponins have been purified by high pressure liquid chromatography (HPLC) and low pressure silica chromatography. These four saponins have adjuvant effect in mice. QA-7, QA-17, QA-18, and QA-21, purified from Superfos "Quil-A," a crude Quillaja saponin preparation, are less toxic in mice than "Quil-A"; QA-17 and QA-18 are less toxic in cats than "Quil-A" (QA-7, QA-21 were not tested). In addition, a toxic component of Superfos "Quil-A" has been identified as QA-19; this component is toxic in mice at lower doses than "Quil-A" or QA-7, QA-17, QA-18, and QA-21. The increased toxicity of QA-19 compared to QA-7, QA-17, QA-18, and QA-21 is unexpected in that this component is a saponin, has a similar carbohydrate composition, exhibits adjuvant activity in mice at doses lower than the toxic dose, and exhibits similar chromatographic behavior. All of the above saponins may be isolated from aqueous extracts of *Quillaja saponaria Molina* bark. The substantially pure saponins of the present invention are useful as immune adjuvants and enhance immune responses in individuals at a much lower concentration than the previously available heterogeneous saponin preparations without the toxic effects associated with crude saponin preparations.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The saponins of the present invention may be obtained from the tree *Quillaja saponaria Molina.*

The term "saponin" as used herein includes glycosidic triterpenoid compounds which produce foam in aqueous solution, have hemolytic activity in most cases, and possess immune adjuvant activity. The invention encompasses the saponin per se, as well as natural and pharmaceutically acceptable salts and pharmaceutically acceptable derivatives. The term "saponin" also encompasses biologically active fragments thereof.

The invention also concerns compositions, such as immunologic compositions, comprising one or more substantially pure saponin fractions, and methods of using these compositions as immune adjuvants.

The term "immune adjuvant," as used herein, refers to compounds which, when administered to an individual or tested in vitro, increase the immune response to an antigen in the individual or test system to which said antigen is administered. Some antigens are weakly immunogenic when administered alone or are toxic to the individual at concentrations which evoke immune responses in said individual. An immune adjuvant may enhance the immune response of the individual to the antigen by making the antigen more strongly immunogenic. The adjuvant effect may also lower the dose of said antigen necessary to achieve an immune response in said individual.

The adjuvant activity of the saponins may be determined by any of a number of methods known to those of ordinary skill in the art. The increase in titer of antibody against specific antigen upon administration of an adjuvant may be used as a criteria for adjuvant activity (Dalsgaard, K. (1978) *Acta Veterinia Scandinavica* 69, 1–40, Scott, M. T., Gross-Samson, M., and Bomford, R. (1985) *Int. Archs. Allergy Appl. Immun.* 77, 409–412). Briefly, one such test involves injecting CD-1 mice intradermally with an antigen (for instance, i.e., bovine serum albumin, BSA) mixed with varying amounts of the potential adjuvant. Sera was harvested from the mice two weeks later and tested by ELISA for anti-BSA antibody. A comparison of the adjuvant effects of the dialyzed, methanolsoluble bark extract and "Quil A" showed that antibody titers were two orders of magnitude greater when the antigen BSA was administered in the presence of the saponin preparations than when BSA was administered in PBS alone. The bark extract possessed good adjuvant activity when administered at an adjuvant dose of 12 μg carbohydrate (assayed by anthrone) or more. The adjuvant response to "Quil-A" was lower than for the bark extract but was evident at doses ranging from 9–23 μg carbohydrate. Carbohydrate weight (determined by assay with anthrone using glucose as a standard) is approximately 30% of the dry weight of these crude adjuvant extracts.

The term "substantially pure" means substantially free from compounds normally associated with the saponin in its natural state and exhibiting constant and reproducible chromatographic response, elution profiles, and biologic activity. The term "substantially pure" is not meant to exclude artificial or synthetic mixtures of the saponin with other compounds.

Preferably, the substantially pure saponin is purified to one or more of the following standards: 1) appearing as only one major carbohydrate staining band on silica gel TLC (EM Science HPTLC Si60) in a solvent system of 40 mM acetic acid in chloroform/methanol/water (60/45/10, v/v/v), 2) appearing as only one major carbohydrate staining band on reverse phase TLC (EM Science Silica Gel RP-8) in a solvent system of methanol/water (70/30, v/v), 3) appearing as only one major peak upon reverse-phase HPLC on Vydac C4 (5 μm particle size, 330 Å pore, 4.6 mm ID×25 cm L) in 40 mM acetic acid in methanol/water (58/42, v/v).

In the preferred embodiment, the saponin adjuvants of the present invention are purified from *Quillaja saponaria Molina* bark. Aqueous extracts of the *Quillaja saponaria Molina* bark were dialyzed against water. The dialyzed extract was lyophilized to dryness, extracted with methanol and the methanol-soluble extract was further fractionated on silica gel chromatography and by reverse phase high pressure liquid chromatography (RP-HPLC). The individual saponins were separated by reverse phase HPLC as described in Example 1. At least 22 peaks (denominated QA-1 to QA-22) were separable. Each peak corresponded to a carbohydrate peak as demonstrated in FIG. 2 and exhibited only a single band on reverse phase thin layer chromatography. The

individual components were identified by retention time on a Vydac C_4 HPLC column as follows:

Peak	Retention Time (minutes)
QA-1	solvent front
QA-2	4.6
QA-3	5.6
QA-4	6.4
QA-5	7.2
QA-6	9.2
QA-7	9.6
QA-8	10.6
QA-9	13.0
QA-10	17.2
QA-11	19.0
QA-12	21.2
QA-13	22.6
QA-14	24.0
QA-15	25.6
QA-16	28.6
QA-17	35.2
QA-18	38.2
QA-19	43.6
QA-20	47.6
QA-21	51.6
QA-22	61.0

Immune adjuvant activity was tested by measuring the ability of the purified saponins to enhance the immune response in mice to exogenously administered antigens. The purified saponins of the present invention demonstrated adjuvant effects at lower doses than the crude extracts. Particularly, the predominant saponins in bark extract (QA-7, QA-17, QA-18, and QA-21) demonstrated adjuvant activity at doses of 4.5 μg carbohydrate or less (assayed by anthrone). The purified saponins were further characterized by carbohydrate content, reverse phase and normal phase TLC, UV, infra red, NMR spectra, and fast atom bombardment—mass spectroscopy.

The approximate extinction coefficient determined for 1% (w/v) solutions in methanol at 205 nm of several of the more preferred purified saponins are as follows:

	1% E_{205} (nm)
QA-7	34
QA-17	27
QA-18	27
QA-21	28

Carbohydrate content was used to quantitate the saponins in some instances. The carbohydrate assay was the anthrone method of Scott and Melvin (*Anal. Chem.* 25:1656 (1953)) using glucose as a standard as described in Example 1. This assay was used to determine a ratio of extent of anthrone reaction (expressed in glucose equivalents) per mg of purified saponin (dry weight) so that dry weight of a particular preparation could be estimated by use of anthrone assay. It must be noted that differences in reactivity with anthrone for different saponins may be due to carbohydrate composition rather than quantity as different monosaccharides react variably in this assay.

The substantially pure QA-7 saponin is characterized as having immune adjuvant activity, containing about 35% carbohydrate (as assayed by anthrone) per dry weight, having a uv absorption maxima of 205–210 nm, a retention time of approximately 9–10 minutes on RP-HPLC on a Vydac C_4 column having 5 μm particle size, 330 Å pore, 4.6 mm ID×25 cm L in a solvent of 40 mM acetic acid in methanol/water (58/42; v/v) at a flow rate of 1 ml/min, eluting with 52–53% methanol from a Vydac C_4 column having 5 μm particle size, 330 Å pore, 10 mM ID×25 cm L in a solvent of 40 mM acetic acid with gradient elution from 50 to 80% methanol, having a critical micellar concentration of approximately 0.06% in water and 0.07% in phosphate buffered saline, causing no detectable hemolysis of sheep red blood cells at concentrations of 200 μg/ml or less, and containing the monosaccharide residues terminal rhamnose, terminal xylose, terminal glucose, terminal galactose, 3-xylose, 3,4-rhamnose, 2,3-fucose, and 2,3-glucuronic acid, and apiose (linkage not determined).

The substantially pure QA-17 saponin is characterized as having adjuvant activity, containing about 29% carbohydrate (as assayed by anthrone) per dry weight, having a UV absorption maxima of 205–210 nm, a retention time of approximately 35 minutes on RP-HPLC on a Vydac C_4 column having 5 μm particle size, 330 Å pore, 4.6 mm ID×25 cm L in a solvent of 40 mM acetic acid in methanol-water (58/42; v/v) at a flow rate of 1 ml/min, eluting with 63–64% methanol from a Vydac C_4 column having 5 μm particle size, 330 Å pore, 10 mm ID×25 cm L in a solvent of 40 mM acetic acid with gradient elution from 50 to 80% methanol, having a critical micellar concentration of 0.06% (w/v) in water and 0.03% (w/v) in phosphate buffered saline, causing hemolysis of sheep red blood cells at 25 μg/ml or greater, and containing the monosaccharide residues terminal rhamnose, terminal xylose, 2-fucose, 3-xylose, 3,4-rhamnose, 2,3-glucuronic acid, terminal glucose, 2-arabinose, terminal galactose and apiose (linkage not determined).

The substantially pure QA-18 saponin is characterized as having immune adjuvant activity, containing about 25–26% carbohydrate (as assayed by anthrone) per dry weight, having a UV absorption maxima of 205–210 nm, a retention time of approximately 38 minutes on RP-HPLC on a Vydac C_4 column having 5 μm particle size, 330 Å pore, 4.6 mm ID×25 cm L in a solvent of 40 mM acetic acid in methanol/water (58/42; v/v) at a flow rate of 1 ml/min, eluting with 64–65% methanol from a Vydac C_4 column having 5 μm particle size, 330 Å pore, 10 mm ID×25 cm L in a solvent of 40 mM acetic acid with gradient elution from 50 to 80% methanol, having a critical micellar concentration of 0.04% (w/v) in water and 0.02% (w/v) in phosphate buffered saline, causing hemolysis of sheep red blood cells at concentrations of 25 μg/ml or greater, and containing the monosaccharides terminal rhamnose, terminal arabinose, terminal apiose, terminal xylose, terminal glucose, terminal galactose, 2-fucose, 3-xylose, 3,4-rhamnose, and 2,3-glucuronic acid.

The substantially pure QA-21 saponin is characterized as having immune adjuvant activity, containing about 22% carbohydrate (as assayed by anthrone) per dry weight, having a UV absorption maxima of 205–210 nm, a retention time of approximately 51 minutes on RP-HPLC on a Vydac C_4 column having 5 μm particle size, 330 Å pore, 4.6 mm ID×25 cm L in a solvent of 40 mM acetic acid in methanol/water (58/42; v/v) at a flow rate of 1 ml/min, eluting with 69 to 70% methanol from a Vydac C_4 column having 5 μm particle size, 330 Å pore, 10 mm×ID 25 cm L in a solvent of 40 mM acetic acid with gradient elution from 50 to 80% methanol, with a critical micellar concentration of about 0.03% (w/v) in water and 0.02% (w/v) in phosphate buffered saline, causing hemolysis of sheep red blood

cells at concentrations of 25 μg/ml or greater, and containing the monosaccharides terminal rhamnose, terminal arabinose, terminal apiose, terminal xylose, 4-rhamnose, terminal glucose, terminal galactose, 2-fucose, 3-xylose, 3,4-rhamnose, and 2,3-glucuronic acid.

The term "individual" means any animal which can elicit an immune response, including humans.

The purified saponins exhibit adjuvant effects when administered over a wide range of dosages and a wide range of ratios to the antigen being administered. In one embodiment, the saponin is administered in a ratio of adjuvant to antigen (w/w) of 3.0 or less, preferably 1.0 or less.

The purified saponins may be administered either individually or admixed with other substantially pure adjuvants to achieve the enhancement of the immune response to an antigen. Among the adjuvant mixtures effective in the present invention are fractions QA-7 and QA-17, QA-7 and QA-18, QA-17 and QA-18, or QA-7, QA-17, and QA-18 administered together. Purified saponins may also be administered together with non-saponin adjuvants. Such non-saponin adjuvants useful with the present invention are oil adjuvants (for example, Freund's Complete and Incomplete), liposomes, mineral salts (for example, $AlK(SO_4)_2$, $AlNa(SO_4)_2$, $AlNH_4(SO_4)$, silica, alum, $Al(OH)_3$, $Ca_3(PO_4)_2$, kaolin, and carbon), polynucleotides (for example, poly IC and poly AU acids), and certain natural substances (for example, wax D from *Mycobacterium tuberculosis*, as well as substances found in *Corynebacterium parvum*, *Bordetella pertussis*, and members of the genus Brucella).

The purified saponins of the present invention may be utilized to enhance the immune response to any antigen. Typical antigens suitable for the immune-response provoking compositions of the present invention include antigens derived from any of the following: viruses, such as influenza, rabies, measles, hepatitis B, hoof and mouth disease, or HTLV-III; bacteria, such as anthrax, diphtheria or tuberculosis; or protozoans, such as *Babeosis bovis* or Plasmodium.

A particular example is the use of the purified saponins of the present invention to enhance the immune response to gp70 recombinant protein. One gp70 recombinant protein is an antigen which contains the polypeptide portion of FeLV gp70 envelope protein. This recombinant antigen is termed "gp70R," "rec-gp70" or "Rgp70." Another antigen preparation which contains the polypeptide portion of FeLV gp70 together with the 40 amino-terminal amino acids (termed "Rgp70delta") or with the entire amino acid sequence (termed "Rgp90") of the p15e envelope protein of FeLV subgroup A is produced using recombinant DNA techniques. These recombinant gp70-containing polypeptides, gp70R, gp70R-delta, and gp90R, are hereinafter referred to collectively as gp70-containing protein. The term gp70-containing protein is intended to include polypeptides having the same amino acid sequence of the naturally occurring gp70-containing protein, and analogs thereof. The term "analogs" is intended to include proteins or polypeptides which differ from gp70, gp70-delta, or gp90 by addition, deletion or substitution of one or more amino acids providing that said polypeptide demonstrate substantially the biological activity of gp70 protein.

Administration of the compounds useful in the method of present invention may be by parenteral, intravenous, intramuscular, subcutaneous, intranasal, or any other suitable means. The dosage administered may be dependent upon the age, weight, kind of concurrent treatment, if any, and nature of the antigen administered. The effective compound useful in the method of the present invention may be employed in such forms as capsules, liquid solutions, suspensions or elixirs for oral administration, or sterile liquid forms such as solutions or suspensions. Any inert carrier is preferably used, such as saline, or phosphate-buffered saline, or any such carrier in which the compounds used in the method of the present invention have suitable solubility properties for use in the method of the present invention.

Having now generally described the invention, the same may be further understood by reference to the following examples, which are not intended to be limiting unless so expressly stated.

EXAMPLE 1

Preliminary Preparation of *Quillaja Saponaria Molina* Bark Extract

Quillaja saponaria Molina bark was stirred with an excess of water (10% w/v) to extract the saponins. The aqueous extract was then filtered and stored in 0.1% NaN_3. 150 ml of this extract was centrifuged at 20,000×g for 30 minutes to remove residual bark fragments. The supernatant, which was light brown, was lyophilized and redissolved in 16 ml of water and the pH was adjusted to less than 4 with the addition of 160 μl of 1N acetic acid. This solution was placed in dialysis tubing having a 12,000 MW cut off and dialyzed against 1 liter of water. The water was changed after 8 hours of dialysis, and the dialysis was allowed to proceed overnight. Samples of the dialysate were removed after the first and second dialysis cycles. The dialyzed extract was lyophilized and extracted with 40 ml methanol at 60° C. for 15 minutes followed by centrifugation at 1,000×g for 10 minutes to sediment the undissolved material. This material was subjected to two additional extractions with methanol. The methanol extracts were pooled, evaporated on a rotoevaporator to dryness, redissolved in 5.5 ml methanol, and filtered through a 0.2μ nylon 66 mesh to remove residual undissolved material. Fractions were analyzed by reverse phase thin-layer chromatography (RP-TLC) on C8 plates (E.M. Science RP-TLC, C8) in a solvent system of 70% methanol/30% water or by normal phase thin layer chromatography on silica gel 60 TLC plates in a solvent system of n-butanol, ethanol, water, and ammonia (30/60/29/21, v/v/v/v). The carbohydrate bands were visualized with Bial's reagent which detected all major bands detectable by sulfuric acid charring with an increased sensitivity over the sulfuric acid charring method. The Bial's reagent carbohydrate stain was routinely used as a detection reagent on TLC plates. All major bands were glycosylated.

Dialysis removed a major carbohydrate-containing band (R_F=0.82 on EM Science RP TLC, C8 in methanol/water (70/30, v/v)), as well as some minor components. In addition, dialysis removed components with strong absorption maxima at 280 and 310 nm. Approximately 80% of the carbohydrate (assayed by anthrone) was removed by dialysis, but about 95% of the hemolytic activity was retained during dialysis.

Most saponin adjuvants are known to have detergent properties, such as hemolysis of red blood cells, so the retention of hemolytic activity is a rough indication of the retention of adjuvant saponins. Several bands were retained by dialysis, indicating their detergent nature.

Methanol solubilized all TLC bands present in the dialyzed extract except one TLC band (R_F=0 on both reverse-phase and silica TLC plates). The methanol-insoluble material was reddish-brown. The material which was methanol-soluble appeared white after lyophilization.

Carbohydrate concentration was determined by the method of Scott and Melvin (Scott, T. A., and Melvin, E. H. *Anal. Chem.* 25, 1656 (1953)). Briefly, an aqueous sample to be tested or glucose as a standard carbohydrate solution (450 μl) was mixed with 900 μl of 0.2% anthrone (w/v) in sulfuric acid and incubated for 16 min at 90°–100° C. The absorbance was read at 625 nm. Glucose was used as a standard.

The hemolytic activity of the samples was determined as follows: Briefly, samples were diluted in a round bottom microtiter plate with 1:2 dilutions in phosphate buffered saline in successive rows (100 μl/well). 10 μl normal rabbit blood in Alsevers solution (Hazelton) was added to each well and mixed. Plates were incubated for one hour at room temperature followed by centrifugation of the plates in a Sorvall RT6000 to sediment unhemolyzed cells. Absence of hemolysis was determined by the presence of a pellet of unhemolyzed cells in the bottom of the well.

EXAMPLE 2

Comparison of Dialyzed, Methanol-Soluble Bark Extract and Superfos "Quil-A" by TLC and HPLC

Superfos "Quil-A" and dialyzed, methanolsoluble components of bark extract prepared as in Example 1 were compared by reverse phase TLC as described in Example 1. All bands present in the bark extract after dialysis and solubilization with methanol were present in "Quil-A." In addition, "Quil-A" contained a band with r_f=0 on reverse-phase TLC plates; this component was removed by methanol-solubilization as described above. The similarity in composition of dialyzed, methanolsoluble bark extract and "Quil-A" was confirmed by HPLC. The individual components of bark extract were separable by reverse-phase HPLC on Vydac C4 (5 μm particle size, 330 Å pore, 4.6 mm ID×25 cm L) in 40 mM acetic acid in methanol/water (58/42, v/v). The refractive index of the individual fractions was determined. FIG. 1 represents the refractive index profile of the peaks (labeled QA-1 to QA-22 in order of increasing retention times) from the RP-HPLC. The relative proportion of each peak in bark extract and Superfos "Quil-A" is shown on Table 1, below.

TABLE 1

Relative proportion of HPLC fractions of crude saponin extract and Superfos "Quil-A" (refractive index) % of Total (peaks 2–21)		
HPLC Fraction	Dialyzed, methanol-soluble bark extract	Superfos "Quil-A"
QA-2	3.1	1.2
QA-3	4.8	2.4
QA-4,5	10.1	7.1
QA-6,7	17.5	12.7
QA-8	6.8	10.5
QA-9	1.0	2.1
QA-10	2.7	1.3
QA-11	6.8	6.2
QA-12	3.5	5.6
QA-13,14,15	4.8	7.7
QA-16	2.8	1.4
QA-17	11.4	9.9

TABLE 1-continued

Relative proportion of HPLC fractions of crude saponin extract and Superfos "Quil-A" (refractive index) % of Total (peaks 2–21)		
HPLC Fraction	Dialyzed, methanol-soluble bark extract	Superfos "Quil-A"
QA-18	13.5	21.8
QA-19	2.2	4.5
QA-20	3.2	2.2
QA-21	5.6	3.7

The individual peaks correspond to single thin-layer chromatography bands on reverse-phase TLC plates. Another representative experiment shown on FIG. 2 demonstrates that the refractive index peaks also correspond to carbohydrate peaks, confirming that all major bark extract components are glycosides (HPLC fractions assayed for carbohydrate by the anthrone assay).

Dialyzed, methanol-soluble bark extract and "Quil-A" were compared directly in this HPLC system. The individual components were identified by retention time. All peaks present in dialyzed, methanol-soluble bark extract were also present in "Quil-A" in similar proportions with the exception of a higher proportion of component QA-8 and a lower proportion of component QA-17 in Superfos "Quil-A" compared to bark extract. FIG. 3 shows a comparison of dialyzed, methanol-soluble bark extract and Superfos "Quil-A" using a semipreparative Vydac C_4 (10 mm ID×25 cm L, 330 Å pore size, 5 μm particle size). The sample is loaded in 50% methanol in 40 nM acetic acid and a methanol gradient in 40 mM acetic acid (shown in FIG. 3) is used to elute the samples. The absorbance was monitored at 214 nm.

Various samples of Quillaja bark were extracted and analyzed by HPLC. There was some variability in the relative proportions of the peaks, but the same peaks were always present. It is not presently known whether the variability in proportions is due to variability in the efficiency of the extraction process or in bark from different sources.

Due to the ready availability of "Quil-A" and the similar composition to bark extract, "Quil-A" was utilized to produce mg quantities of material. Adjuvant activity in mice, using BSA as antigen, was found to be associated with peaks 4, 7, 11, 12, 15, 16, 17, 18, 19, and 20 (Table 2) at doses of 3.0 μg carbohydrate (determined by the anthrone assay). The absorbance due to antigen-specific antibody binding (two weeks post-immunization, determined by ELISA) at a sera dilution of 1:10 provides a semi-quantitative estimate of adjuvant activity (ranging from 0.07 in mice immunized in the absence of adjuvant to 1.24 in mice immunized in the presence of QA-20).

TABLE 2

Adjuvant Activity in Mice		
HPLC Fraction	Adjuvant Dose (μg carbohydrate)	Absorbance* (410 nm)
QA-2	3.0	.34
QA-3	3.0	.27
QA-4	3.0	.60
QA-7	3.0	.49
QA-10	3.0	.13
QA-11	3.0	.46
QA-12	3.0	.76
QA-13,14	3.0	.20
QA-15	3.0	1.17

TABLE 2-continued

HPLC Fraction	Adjuvant Activity in Mice: Adjuvant Dose (μg carbohydrate)	Absorbance* (410 nm)
QA-16	3.0	.66
QA-17	3.0	1.13
QA-18	3.0	.75
QA-19	3.0	.93
QA-20	3.0	1.24
		0.07

*Absorbance due to antigen-specific antibody binding at sera dilution of 1:10.

Due to the predominance of peaks QA-7, QA-17, QA-18, and QA-21 in bark extract, these four components were purified on a layer scale, as described in Examples 3 and 4, below.

EXAMPLE 3

Purification by Silica Chromatograph

1 gram "Quil-A" was suspended in 75 ml methanol and heated at 60° for 15 minutes and filtered. The undissolved material was extracted a second time with 50 ml methanol at 60° C. and filtered. The filtrates were evaporated to dryness on the rotoevaporator. A Lichropep Silica Si60 column (E.M. Science, 25 mm ID×310 mm L, 40–63 μm particle size) was pre-equilibrated in 40 mM acetic acid in chloroform/methanol/water (62/32/6, v/v/v).

The dried "Quil-A," a crude mixture of saponins, was dissolved in 5 ml of column solvent and eluted through the silica isocratically in this solvent system at a flow rate of 1 ml/min. Carbohydrate analysis, thin-layer chromatography, and HPLC were used to monitor the fractions for QA-7, QA-17, QA-18, and QA-21. Fractions 19–30 were enriched in QA-21 and were pooled for further purification of QA-21. Fractions 31–60 were enriched in QA-8 and QA-18 and were pooled for further purification of these components. Fractions 85–104 were enriched with QA-7 and QA-17 and were pooled for further purification of these components. These pools were flash evaporated prior to further purification.

EXAMPLE 4

Further Purification by Reverse Phase HPLC

Silica fractions were further purified by semipreparative reverse phase HPLC on Vydac C_4 (10 mm ID×25 cm L), FIG. 4. Silica fractions (10–20 mg) were dissolved in the appropriate solvent and loaded on Vydac C_4. A methanol gradient was used to elute the fractions. The flow rate was 3 ml per minute. The fractions were monitored by absorbance at 214 nm. FIG. 4B shows the purification of QA-21 from silica fractions 19–30 using isocratic separation in 40 mM acetic acid in 58% methanol/42% water. Fractions eluting with a retention time between 65–72 minutes were identified as QA-21 by reverse phase TLC and pooled for further characterization. FIG. 4C shows the purification of QA-18 from silica fractions 31–60 using a methanol gradient in 40 mM acetic acid (50–56% methanol/0–10 min, 56–69% methanol/10–79 min). Fractions eluting with a retention time between 46–48 minutes were identified as QA-18 by reverse phase TLC and pooled for further characterization. FIG. 4D shows the purification of QA-7 and QA-17 from silica fractions 85–104 using the same gradient used in FIG. 4C. Fractions eluting with a retention time between 21–23 minutes were identified as QA-17 by reverse phase TLC and pooled for further characterization. Fractions eluting with a retention time between 44–46 minutes were identified as QA-17 by reverse phase TLC and were pooled for further characterization.

EXAMPLE 5

Purity and Characterization of Adjuvants Purified by Silica and Reverse Phase Chromatography

Purity

FIG. 5*a* represents a reverse-phase TLC (E.M. Science RP-TLC, C8 (Solvent=70% methanol, visualization spray=Bial's reagent)). 5 μg each of QA-7, QA-17, QA-18, and QA-21 purified as described in Example 3 and 4, were chromatographed. The adjuvants each appeared as single bands in this TLC system.

FIG. 5*b* demonstrates fractions QA-7, QA-17, QA-18, QA-21 and "Quil-A" on EM Si60 HPTLC plate (solvent=40 mM acetic acid in chloroform/methanol/H_2O (60/45/10, v/v/v), visualization spray=Bial's reagent). 2 μg each of QA-7, QA-17, QA-18 and QA-21, purified as described in Examples 3 and 4, and 20 μg of "Quil-A," a crude saponin extract, were chromatographed. The HPLC-purified material appeared predominantly as a single band.

Spectroscopy

The UV spectra of QA-7, QA-17, QA-18 and QA-21 in methanol are shown on FIGS. 6A–D respectively. Dalsgaard's (Dalsgaard, K., *Acta Veterinaria Scandinavica Supp.* 69:1–40 (1978)) adjuvant fraction had an absorbance peak at 280 nm; however, the HPLC-purified fractions of the present invention do not have a peak at 280 nm but have a major peak in the region between 200–220 nm with a shoulder centered at 260 nm.

Fourier Transform-Infrared Resonance ("FT-IR") spectra showed little difference between the adjuvants, suggesting that they all have the same functional groups. Although identification of the structure cannot be made from the IR, the spectral data is consistent with the presence of a carboxyl group as was suggested by Dalsgaard (Dalsgaard, K., supra).

^{1}H-NMR at 250 MHz of the purified saponins in CD_3OD demonstrates the complex nature of the purified saponins QA-7 (FIG. 7A), QA-18 (FIG. 7B), and QA-21 (FIG. 7C). The signals in the region between 4.1 to 5.4 ppm clearly demonstrate the presence of multiple signals from the anomeric protons of the monosaccharides, indicating a multiplicity of monosaccharide resides. However, the NMR spectra of the saponins are too complex to allow structural determination.

MS-FAB of the purified saponins QA-7, QA-17, and QA-21 (FIGS. 8A, 8B, 8C, respectively) indicated approximate pseudo- molecular ion masses of 1870, 2310, and 1980, respectively. MS-FAB was not determined on QA-18 due to difficulties in solubilizing this component. These molecular weights are consistent with those expected for a triterpene linked to eight to ten monosaccharide residues and were in the same range as monomer molecular weights determined by size exclusion HPLC of purified saponins in methanol (Zorbax PSM 60 Si column, 25 cm×6.2 mm, 1 ml/min flow rate, molecular weight standards=18-β-glycrhetinic acid and ginenoside Rb_1) which indicated approximate molecular weights of 2600, 2400, 1800, and 2400 for QA-7, QA-17, QA-18, and QA-21, respectively. The difference between FAB-MS and size exclusion HPLC are

most likely due to variation in shape between the saponins and the molecular weight standards.

Carbohydrate Composition

Table 3 below shows the carbohydrate composition and linkage analysis of purified saponins QA-7, QA-17, QA-18, QA-21, and QA-19. The carbohydrate in saponins was converted to alditol acetates by heating 0.2 mg saponin in 0.3 ml 2N trifluoroacetic acid containing 0.1 mg/ml inositol at 120° C. for two hours. The acid was removed under a flow of air, and residual acid removed by the addition of isopropanol (2×0.25 ml), followed by blowing to dryness with air. The dry residue obtained was dissolved in 1M ammonium hydroxide (0.25 ml) containing 10 mg/ml sodium borodeuteride and kept for one hour at room temperature. Glacial acetic acid (0.1 ml) was added, and the solution was blown to dryness. Residual borate was removed by co-distilling with 10% acetic acid in methanol (3×0.25 ml) and finally with methanol (2×0.25 ml). The dry residue in acetic anhydride (0.1 ml) and pyridine (0.1 ml) was heated for 20 minutes at 120° C. Toluene (9.02 ml) was added to the cooled solution, and the solvents removed under a flow of air. This procedure of adding toluene and removing pyridine and acetic anhydride was repeated twice. The residue obtained was taken up in dichloromethane (0.5 ml) and extracted with water (0.5 ml). The organic phase was transferred to a clean tube and dried. Prior to analysis by GLC (gas-liquid chromatography), the residue was dissolved in acetone (0.1 ml). Alditol acetates were analyzed on an SP2330 capillary GLC column (30 m×0.25 mm) at 235° C.) with flame ionization detection. The carbohydrate in saponins was converted to trimethylsilated methylglycosides by heating 0.1 mg of sample in methanolic HCl (0.3 ml) containing 50 ug/ml inositol for 16 hours at 80° C. The sample was blown to dryness, and residual acid removed by the addition of t-butyl alcohol (2×0.25 ml) followed by drying with a flow of air. The dry residue was dissolved in a solution (0.2 ml) containing pyridine, hexamethyldisilazane, and trimethylchlorosilane (5:1:0.5 v/v, "Tri-Sil") and heated for 20 minutes at 80° C. The silylating reagent was evaporated at room temperature, and the residue dissolved in hexane (1 ml). After removal of the insoluble residue by filtration using glass wool plug, the filtrate was transferred to a clean tube and evaporated. The residue was dissolved in hexane (0.2 ml) prior to analysis by GLC. The trimethylsilated methyl glycosides were analyzed on a GLC column of fused silica DB1 (25 m×0.25 mm) for 3 min at 160° C. followed by a 2°/min increase to 200° C. and then a 10°/min increase to 260° C. with flame ionization detection.

Glycoside linkage analysis was carried out by the following method: To the sample (≈1 mg) dissolved in dry dimethylsulfoxide (0.2 ml), 0.2 ml of potassium dimethylsulphinyl anion (2M) was added, and the mixture stirred for 12 hours under argon. The reaction mixture was cooled in ice, and methyl iodide (0.2 ml) was added drop wise. The resulting mixture was sonicated and stirred at room temperature for one hour. The methylated material was isolated using Sep-Pak C_{18} cartridges conditioned with ethanol (20 ml), acetonitrile (8 ml), and water (10 ml). Water (1 ml) was added to the methylation reaction mixture, and the excess methyl iodide removed by passing nitrogen through the solution. The clear solution was applied to the cartridge which was washed with water (8 ml) and 20% acetonitrile (5 ml). The methylated material was eluted from the cartridge with 100% acetonitrile (4 ml) and ethanol (4 ml). The solvents were removed with a flow of air. The dried methylated material was treated with 0.3 ml of "super deuteride" solution at room temperature for one hour in order to reduce the uronic acid residues to the corresponding hexoses. After destroying the excess reagent with glacial acetic acid (0.1 ml), the reaction mixture was blown to dryness with 10% acetic acid/-methanol and blown to dryness two more times. The resulting reduced methylated material in methanol was passed through a column of Dowex—50 W(H+) and the effluent obtained was dried. The reduced methylated material was converted to methylated alditols as described in section 1 above and analyzed by GLC (SP2330 fused silica column (30 m×0.25 mm), 3 min at 170° C. followed by 4°/min to 240° C.) and GLC-MS (SP2330 fused silica column (30 m×0.25 mm), 2 min at 80° C. followed by 30°/min to 170° C. followed by 4°/min to 240° C. followed by holding at 240° C. for 10 min, mass spectral analysis on Hewlett-Packard MSD).

Despite the similarity in the carbohydrate composition, subtle differences distinguish the individual saponins, in particular, the absence of arabinose in QA-7 and decreased glucose in QA-21 compared to the other saponins.

TABLE 3

Carbohydrate Composition and Linkage Analysis of Purified Saponins

	QA-7			QA-17			QA-18			QA-19A			QA-21		
	AA[a]	TMS[b]	Linkage	AA	TMS	Linkage	AA	TMS	Linkage	AA	TMS	Linkage	AA	TMS	Linkage
rhamnose	191.4	1.57	T[c] 3,4	184.8	1.9	T 3,4	132.0	0.99	T 3,4	32.7	1.69	T 3,4	131.9	1.07	T 4
fucose	86.7	0.67	2,3	77.9	0.78	2	95.6	0.76	2	26.6	0.88	2	99.8	0.76	2
arabinose	trace	trace		65.4	0.80	2	80.1	0.64	T	31.1	0.94	T	71.0	0.65	T
xylose	98.1	0.95	T 3	81.8	1.08	T 3	117.8	1.16	T 3	49.9	2.07	T 3	114.3	1.21	T 3
galactose	81.2	0.74	T	69.4	0.81	T	88.1	0.86	T	trace	1.11	T	88.1	0.84	T
glucose	81.2	1.0	T	85.7	1.0	T	89.2	1.00	T	trace	1.0	T	19.6	0.30	T
glucuronic acid	N.T.[d]	0.48	2,3	N.T.	0.52	2,3	N.T.	0.62	2,3	29.2	0.62	2,3	N.T.	0.62	2,3
apiose[e]	22.5	N.T.		24.5	N.T.		25.7	N.T.	T	27.7		T	20.0	N.T.	T

[a]Alditol acetate (μg/mg saponin)
[b]Trimethylsilated methyl glycosides (relative proportions)
[c]T-terminal glycosyl residue, that is, attached through C-1 but with no other residues attached to it. 3,4 = a glycosyl residue attached through C-1 with other glycosyl residues glycosidically attached to it through C-3 and C-4.
[d]Not tested
[e]Poor recovery as alditol acetates

Characterization of Saponins as Detergents

The critical micellar concentration of adjuvants QA-7, QA-17, QA-18, and QA-21 was determined by the method of DeVendittis et al. (DeVendittis, E., Palumbo, G., Parlato, G., and Bocchini, V. (1981) *Anal.*

Biochem. 115, 278–286) as follows: The emission spectrum of 1-anilinonapthalene-8-sulfonic acid (ANS) in water was determined at dry weight concentrations of adjuvant ranging from 0.01 to 0.10% (W/v) to cover the range below and above the critical micellar concentration. Above the critical micellar concentration, the fluorescence yield of ANS increases and the wavelength of maximum emission decreases due to partitioning of the fluorescent dye into the micelles. Similar critical micellar concentrations were found for QA-7, QA-17, QA-18, and QA-21 in water (0.06%, 0.06%, 0.04%, and 0.03%, respectively) with slightly lower concentrations determined in phosphate buffered saline (0.07% 0.03%, 0.02%, and 0.02%, respectively).

FIG. 9 shows the gel filtration chromatograph for micelles formed by purified QA-18 and QA-21 (on Bio-Gel P-200 (6.6 mm ID×90 cm ht)), pre-equilibrated in a concentration of purified saponin equivalent to the critical micellar concentration of that saponin in phosphate buffer saline to prevent the monomer-micelle equilibrium from reducing the apparent radius of the micelles). QA-18 and QA-21 micelles elute with a size that is similar to that of the protein bovine serum albumin.

The hemolytic activity of the adjuvants was determined by the following method: Dilutions of adjuvants QA-7, QA-8, QA-17, QA-18, QA-21, and Superfos "Quil-A" were made on a round bottom , microtiter plate (75 μl per well). Sheep red blood cells (SRBC), washed three times with PBS, were diluted to 4% with PBS. SRBC (25 μl) were added to each well and mixed with adjuvant. After incubation at room temperature 30 min, the plates were spun at 1000 rpm 5 min in a Sorvall RT6000, H-1000 rotor, to sediment unhemolyzed cells. 50 μl of the supernatant from each well was transferred to the same well of a flat bottom microtiter plate and diluted to 200 μl with H_2O. Absorbance was determined at 570 nm with a Dynatech microtiter plate reader. (FIG. 9) Hemolysis increased the absorbance at 570 nm due to release of hemoglobin from the lysed cells. Significant differences in hemolysis were observed between adjuvants. QA-17, QA-18, QA-21, and Superfos "Quil-A" caused partial hemolysis at concentrations as low as 25 μg/ml whereas partial hemolysis was observed with QA-8 at 150 μg/ml. No hemolysis was observed with QA-7 at the concentrations tested (200 μg/ml and less).

EXAMPLE 6

Isolation of Toxic Component QA-19

The toxic component QA-19 cochromatographs with QA-18 on silica and is enriched in silica fractions 31–60. These fractions were pooled and flash evaporated prior to further purification. FIG. 4C shows the separation of QA-19 from QA-18 by reverse phase HPLC on Vydac C_4 (10 mm ID×25 cm L) using a methanol gradient. Fractions eluting with a retention time between 50–52 minutes were identified as QA-19 by reverse phase TLC and analytical HPLC and pooled for further characterization. QA-19 could be further separated into two peaks by repurification in a shallower methanol gradient, with the peak with shorter retention time designated QA-19a and the peak with longer retention time designated QA-19b. Carbohydrate analysis of peak QA-19a which is more toxic in mice than QA-19b, shows a carbohydrate composition which is similar to that of the other saponins (Table 3).

EXAMPLE 7

Isolation of Alkaline Hydrolysis Product

Treatment of QA-18 by brief alkaline hydrolysis yielded one major carbohydrate-containing alkaline hydrolysis product (designated QA-18 H). Purified QA-18 H was prepared from QA-18 and isolated in the following manner:

One ml QA-18 (5 mg/ml) was incubated with 25 μl 1N NaOH for 15 minutes at room temperature. The reaction was stopped with the addition of 100 μl 1N acetic acid. Using these hydrolysis conditions, QA-18 was completely converted to a major hydrolysis product (QA-18 H) eluting in a peak with retention time of 8.0 min compared to 66.8 min for unhydrolyzed QA-18, indicating the increased hydrophilicity of QA-18 H. (Chromatography on Vydac C_4 (4.6 mm ID×25 cm L) in 0.1% trifluoroacetic acid in 55/45 methanol/water v/v) and eluted in a gradient to 64/36 methanol/water (v/v) over 180 minutes, flow rate of 1 ml/minute). The peak containing pure QA-18 H (retention time 8.0 min) was pooled for further characterization. The hydrolysis product of QA-21, designated QA-21 H, was prepared and purified in the same manner. QA-21 H had a retention time of 9.3 minutes compared to 80.4 minutes for unhydrolyzed QA-21. These hydrolysis products were shown by retention time on HPLC and by reverse phase thin layer chromatography to be identical to the major hydrolysis products generated using the method of Higuchi et al., *Phytochemistry* 26: 229 (1987) using mild alkaline hydrolysis in NH_4HCO_3 (Table 4). In addition, these products, QA-18 H and QA-21 H, were shown to be the major breakdown products from hydrolysis of "Quil-A", a crude saponin mixture containing QA-7, QA-17, QA-18, and QA-21 as well as other saponins, indicating that the hydrolysis products QA-21 H and QA-18 H are the same hydrolysis products isolated by Higuchi et al., supra, for structural characterization. QA-18, H and QA-21 H were saved for further characterization of adjuvant activity.

TABLE 4

Retention Time of Major Alkaline Hydrolysis Products	
QA-17 H	8.0[a]
QA-18 H	8.0[a]
	8.2[b]
QA-21 H	9.3[a]
	9.5[b]
Hydrolyzed - "Quil-A"	8.2[a], 9.3[a]

[a]Cambridge BioScience hydrolysis conditions: 5 mg/ml saponin, pH 13. reaction time = 15 minutes at room temperature
[b]Higuchi et al. hydrolysis conditions: 5 mg/ml saponin, 6% NH_4HCO_3, methanol/-H_2O (1/1, v/v), reaction time = 60 minutes at 100° C.
HPLC Conditions:
Vydac C^4, 5 μm particle size, 300 Å pore size, .46 × 25 cm
Solvent A = 0.1% trifluoroacetic acid in water
Solvent B = 0.1% trifluoroacetic acid in methanol
Gradient = 55–64% B/180 minutes
Flow rate = 1 ml/min

EXAMPLE 8

Testing for Adjuvant Effect Using BSA as Antigen

Briefly, adjuvant effect is assessed by increase in antigen-specific antibody titers due to addition of potential adjuvant in the immunization formulation. Increased titers result from increased antibody concentrations and/or increased antigen/antibody affinity. Adjuvant effects of saponins have previously been measured by increase in titer of neutralizing antibodies to foot-and-

mouth disease vaccines in guinea pigs (Dalsgaard, K., *Archiv. fur die gesamte Virusforschung* 44, 243–254 (1974)), increase in titer of precipitating antibodies to BSA (as measured by radial immunodiffusion) in guinea pigs vaccinated with BSA/saponin mixtures (Dalsgaard, K. *Acta Veterinaria Scandinavica* 69, 1–40 (1978)), as well as by the increase in titer of anti-keyhole limpet hemocyanin (KLH) antibody (measured by ELISA) in mice immunized with KLH/saponin (Scott, M. T., Gross-Samson, and Bomford, R., *Int. Archs. Allergy Appl. Immun.* 77:409–412 (1985)).

Assessment of adjuvant effect in this study was determined by increase in anti-BSA antibody following immunization with BSA/saponin compared with immunization with BSA in the absence of saponin. The adjuvant activity in the purified fraction was measured as follows: CD-1 mice (8–10 weeks old) were immunized intradermally with the following formulation: 10 μg BSA (Sigma 7030, fatty acid free) and Quillaja adjuvant (at doses ranging from 1.5–45 μg carbohydrate as measured by anthrone) in 200 μl PBS. Sera was harvested two weeks post-immunization. Anti-BSA antibody was determined by ELISA: Immulon II plates were coated overnight at 4° C. with 100 μl fatty acid free BSA (10 μg/ml in PBS) in rows, A, C, E, and G. Plates were washed twice with PBS. Nonspecific binding was prevented by incubating for 1.5 h at 37° C. with 100 μl diluent (2% Casein acid hydrolysate (Oxoid, w/v) in PBS) per well in all wells. Plates were washed four times with 0.05% Tween 20 in distilled water. Sera at dilutions of 10, 10^2, 10^3, and 10^4 were incubated in rows A+B, C+D, E+F, and G+H, respectively (100 μl/well) for 1 h at room temperature. Plates were washed as described above. Boehringer-Mannheim horse radish peroxidase conjugate goat anti-mouse antibody (1/5000 in 5% BSA in diluent) was incubated for 30 min at room temperature (100 μl per well, all wells). Plates were washed as described above. The extent of peroxidase reaction was determined by reaction with 2,2'-azino-bis(3-ethylbenzthiazoline)-6-sulfonate (30 minute reaction at room temperature, absorbance measured at 410 nm) or with 3,3',5,5'-tetramethylbenzidine (10 min reaction at room temperature, absorbance measured at 450 nm). The contribution of nonspecific antibody binding to the total antibody binding was removed by subtraction of the absorbance of the antigen-negative well from the absorbance of the antigen-positive well for each sera dilution. The absorbance due to antigen-specific binding was plotted as a function of the logarithm of the sera dilution. (FIG. 11) Typical endpoint titers were typically at a sera dilution of 10 or less for immunization in the absence of adjuvant and were as high as 10^3 in the presence of saponin adjuvant. Dialyzed, methanol-soluble bark extract at an adjuvant dose of 12 μg carbohydrate or greater (carbohydrate assayed by anthrone) increased titers by 2 orders of magnitude compared to BSA in PBS. A good adjuvant effect was observed at doses of "Quil-A" between 9–23 μg carbohydrate.

EXAMPLE 9

Adjuvant Testing of HPLC-Purified Extract Components

By the criteria described in Example 8, peaks QA-7, QA-11, QA-12, QA-15, QA-16, QA-17, QA-18, QA-19, and QA-20 have varying degrees of adjuvant effect with QA-15, QA-17, QA-18, QA-19, and QA-20 being particularly effective at a dose of 3.0 μg carbohydrate in this particular experiment. Due to the small number of mice used per immunization (2) and the natural variation in immune response between individual mice, this experiment cannot be used to quantitatively assess the relative adjuvant effect of these peaks. However, it provides a qualitative assessment of the presence of adjuvant activity. It must also be noted that the absence of apparent effect with QA-2, QA-3, QA-10, QA-13, and QA-14 does not rule out an adjuvant effect at different adjuvant doses or adjuvant/protein ratio.

Further adjuvant studies were carried out with QA-7, QA-17, and QA-18 at different protein/adjuvant ratios. In general, a good adjuvant effect was observed for QA-7, QA-17, and QA-18 when used at protein/adjuvant ratios (protein weight/carbohydrate weight) of approximately 3:1 to 9:1 (FIG. 12). QA-21 (tested in this study only at protein/carbohydrate weight of 6:1) also showed an adjuvant effect. However, it should be noted that the proper adjuvant to protein ratio for optimum immune response is a function of both the particular saponin adjuvant and the particular antigen used. Adjuvant association with antigen plays an important role in the mechanism of action of the saponin adjuvant effect. In the case of saponin binding to protein, hydrophobic interactions are the predominant factor. Hence, differences in hydrophobicity of the HPLC-purified adjuvants will affect the binding constant to hydrophobic proteins. In addition, the number of hydrophobic binding sites on the protein will also affect the ability to associate with saponin adjuvants. Hence, it is necessary to determine the optimum adjuvant dose for each individual adjuvant and antigen. Such optimization is within the skill of the art.

HPLC-purified adjuvants were also compared with Freund's complete adjuvant and were found to result in a similar level of immune response (FIG. 12, panel b).

EXAMPLE 10

Preparation of FELV Recombinant gp70R-delta

Inclusion Body Preparation

Recombinant *E. coli* clone R16-38 was grown in LB medium supplemented with 1% glucose and 0.1% casamino acids at 32° C. to an optical density (560 nm) of 0.4–0.6. The culture was then shifted to 42° C. and incubated for an additional 2 hours. At the end of this time the cells were collected by centrifugation at 4,000 g for 30 minutes, washed with 50 Tris HCl, pH 7.5, and finally resuspended in 200 ml 50 Tris HCl to which is added 1 ml 0.1M phenylmethylsulfonylfluoride in isopropanol (final concentration 0.5 and 0.4 ml of 5 mg/ml aprotinin (final concentration = 10.0 ug/ml). The cells were lysed by enzymatic digestion with lysozyme (final concentration = 0.5 mg/ml) in the presence of 0.2% Triton X-100. After stirring for 30 minutes, 2 ml $MgCl_2$ (0.5M), 5 ml DNaseI (1 mg/ml) and 1 ml 0.1M phenylmethylsulfonylfluoride were added. After stirring for 30 additional minutes, 40 ml EDTA (0.25M, pH 7.5) and 4 ml Triton X-100 (10% w/v) were added. The preparation was centrifuged at 10,000×g for 30 minutes at 4° C., and the pellet was resuspended in 50 ml 50 Tris HCl, pH 7.5. The pellet was homogenized at low speed for 15 seconds. Lysozyme was added to a concentration of 0.5 mg/ml and 0.6 ml of 10% Triton X-100 were added. After stirring for 15 minutes, 10 ml of $MgCl_2$ (0.5M) and 1 ml DNase I (1 mg/ml) were added and stirring was continued for an additional 15 minutes. After adjusting the volume to 300 ml with 50 Tris, pH 9.0, 40 ml of 10%

Triton X-100 and 51.2 ml of EDTA (0.25M, pH 7.5) were added and the final volume adjusted to 400 ml with 50 Tris, pH 9.0. After stirring for 30 minutes, the suspension was centrifuged at 10,000×g for 30 minutes at 4° C., and the pellet was resuspended in 400 ml 50 Tris HCl, pH 7.5, containing 4M urea, 50 EDTA, and 1% Triton X-100. After stirring for 15 minutes, the suspension was centrifuged at 10,000×g for 30 minutes at 4° C., and the pellet was resuspended in 400 ml 50 Tris HCl, pH 7.5, containing 1.0M NaCl. After stirring for 15 minutes, the suspension was centrifuged at 10,000×g for 30 minutes at 4° C., and the pellet was resuspended in 400 ml 50 Tris HCl, pH 7.5, containing 6M urea, and 5 EDTA. After stirring for 15 minutes, the suspension was centrifuged at 10,000×g for 30 minutes at 4° C. At this point the pellet of inclusion bodies was either frozen for future use or solubilized in 50 Tris HCl, pH 9.5, containing 6M guanidine HCl, 50 EDTA, and 0.5% beta-mercaptoethanol. The gp70R-delta polypeptide was then purified by either of the methods of Example 11, below.

EXAMPLE 11

Purification of FeLV Recombinant gp70R-delta

Procedure I

The solubilized protein of Example 8 was dialyzed against 6M urea, 50 Tris-Cl, pH 8.0, 5 EDTA, and 1 dithiothreitol (DTT). Approximately 120 mg of the protein was applied to a CM-TSK column (EM Science, 1.5 cm ID×4 cm) equilibrated with the same buffer. The protein was eluted with a linear gradient of NaCl (0–1.0M in 150 ml) in the same buffer. The fractions were collected and analyzed by electrophoresis on 10% SDS-polyacrylamide gels. Coomassie-staining was used to identify the gp70R-delta protein. Fractions 25–31, eluting at approximately 0.1M NaCl, were pooled and used for immunization.

Procedure II

In order to decrease the hydrophobicity of gp70R-delta, the sulfhydryl groups were alkylated with iodoacetamide and the lysine residues were N-acylated with citraconic anhydride. The protein prepared as in Example 8 was solubilized in 6M guanidine-HCl in 50 mM borate, pH 9.0, 0.5% beta-mercaptoethanol (v/v). Iodoacetamide is added at a molar ratio of 1:1 (iodoacetamide:total sulfhydryl groups). The alkylation was carried out in the dark for 1 hour at room temperature. The alkylation of all sulfhydryl groups (in the protein and beta-mercaptoethanol) was monitored with DTNB (Ellman's reagent) to ensure complete alkylation. The protein concentration was adjusted to 2 mg/ml.

The protein was citraconylated in the dark by the addition of citraconic anhydride (0.0022 ml per mg protein; approximately 50 molar excess over free lysines). The preparation was dialyzed several times in the dark against 50 mM borate, pH 9.0. The completion of the acylation of the protein lysine groups was determined by reaction with trinitrobenzene sulfonic acid (TNBS) which measures residual free lysine groups. TNBS (200 µl of 10 mM) was added to 200 µg alkylated, citraconylated, dialyzed gp70R-delta in 1 ml 50 mM sodium borate, pH 9.0. The mixture was incubated for 2 hours in the dark at 40° C., the reaction quenched with 0.5 ml of 1N HCl and 0.5 ml 1% SDS, and the absorbance was read at 340 nm. The concentration of TNP-lysine was determined using a molar extinction coefficient of 10,400.

The purification of the alkylated, citraconylated gp70R-delta was performed at pH 9.0 to prevent deblocking of lysine groups. Urea at a final concentration of 4M was added to the modified protein. The protein was concentrated to 3 mg/ml by ultrafiltration and applied to a Sepharose 6B-Cl column (1.5×86 cm). The gp70R-delta protein was eluted at a flow rate of 6.6 ml/hr with 4M urea, 50 mM sodium borate, pH 9.0. Fractions (5.3 ml/fraction) were collected and the gp70R-delta was determined by protein assay and SDS-polyacrylamide electrophoresis to be in fractions 13–15.

The citraconylation of gp70R-delta was reversed by dialyzing 5 ml of alkylated, citraconylated gp70R-delta (1.0 mg/ml) against 6M urea in 50 mM sodium citrate, pH 5.5 for 48 hours at room temperature. The gp70R-delta was dialyzed against 6 M urea in 100 mM sodium bicarbonate, pH 8.0 and the protein concentration adjusted to 0.8 mg/ml prior to absorption to aluminum hydroxide.

Procedure III

A modification of the above purification of alkylated, citraconylated gp70R-delta was developed. Briefly, alkylated, citraconylated gp70R-delta is modified and dialyzed against 50 mM sodium borate, pH 9.0 as described above. Urea was added to a final concentration of 8.0M. The protein was concentrated by ultrafiltration with a PM-30 membrane to yield 2.5 mg protein/ml. The protein solution was applied to a Sephacryl S-400 column (1.5×90 cm) in a 50 mM sodium borate buffer, pH 9.0 containing 8M urea and eluted with the same buffer. Fractions (2.9 ml/fraction) were collected and fractions 34–37 containing gp70R delta were pooled. Twenty-one mg of the protein from these fractions were diluted to a final concentration of 4M urea with 50 mM sodium borate, pH 9.0 and applied to a DEAE-TSK column (1.5×11 cm). The protein was eluted with a linear gradient of NaCl (0–0.5M) in 50 mM sodium borate, pH 9.0 containing 4M urea. Three ml fractions were collected. Fractions 89–95 containing gp70R-delta were pooled and 15 mg of gp70R-delta was recovered.

EXAMPLE 12

Immunization with Aluminum Hydroxide-Absorbed gp70R-delta

Aluminum hydroxide which has been found to have an adjuvant effect for many proteins and is coolly used in vaccines was used as a carrier for gp70R-delta. gp70Rdelta prepared by procedure I of Example 11 above absorbs tightly to 10% aluminum hydroxide in the presence of 50 mM Tris-Cl, pH 8.0 containing 6M urea. Approximately 3 µg gp70R-delta were absorbed per 100 µg aluminum hydroxide. The gp70R-delta absorbed to the aluminum hydroxide was washed with phosphate buffered saline (PBS), resuspended in PBS and used for immunization of animals.

CD-1 mice (8–10 weeks old) were immunized intradermally with gp70R-delta absorbed to $Al(OH)_3$ in a total volume of 200 µl PBS in the presence and absence of HPLC-purified saponins QA-17 or QA-18 or a mixture of QA-17 and QA-18. Twenty to twenty-five µg of gp70R-delta were injected per dose. HPLC-purified saponins QA-17 or QA-18 or a mixture of QA-17 and QA-18 were used at a dry weight dose of 10 µg. Two mice were injected for each formulation. Mice were given a booster injection of gp70R-delta/aluminum

hydroxide six weeks after the initial injection. Mouse sera was analyzed for reactivity to FEA, a FeLV subgroup A, at 2, 4, and 8 weeks post-immunization by an ELISA immunoassay. Four weeks following immunization, an anti-FeLV response elicited by the recombinant gp70-delta was observed. HPLC-purified saponin adjuvants QA-17 and QA-18 boost this response. The response was two orders of magnitude greater at four weeks post-immunization in the presence of QA-17 compared to immunization in the absence of saponin adjuvant. The results of this experiment are shown in FIG. 13.

Anti-FEA antibody was assayed by an ELISA assay. FEA virus (10 μg/ml in PBS) was absorbed to Immulon II plates overnight at 4° C. (100 μl/well). The plates were washed with PBS and nonspecific antibody binding was blocked by incubation for 1 hour with 10% normal goat serum in PBS (100 μl/well) at room temperature. Plates were then washed with 0.05% Tween-20 in distilled water. Sera was diluted in 10% normal goat serum in PBS and incubated for 1 hour at room temperature on the plate at serum dilutions of 10, 10^2, 10^3, and 10^4 (100 μl/well). After washing the plates with 0.05% Tween-20 in distilled water, they were incubated for 30 minutes at room temperature with 100 μl/well of peroxidase-conjugated goat anti-mouse IgG (Boehringer-Mannheim) diluted 1/5000 in PBS. After washing the plates with 0.05% Tween-20 in distilled water, the amount of IgG-binding was determined by peroxidase reaction with 3,3',5,5'-tetramethylbenzidine from the absorbance at 450 nm determined on a Dynatech microliter plate reader.

EXAMPLE 13

Immunization with Aluminum Hydroxide-Absorbed Alkylated gp70R-delta

CD-1 mice (8–10 weeks old) were immunized intradermally with 15 μg/dose of alkylated gp70R-delta purified by procedure II of Example 11 (absorbed to aluminum hydroxide as described in Example 12) in 200 μl PBS. HPLC-purified adjuvants AQ-7, AQ-17, AQ-18 and mixtures of the three adjuvants were used at a dry weight dose of 10 μg. Three mice were injected for each formulation. Mouse sera was analyzed by ELISA at 2 and 4 weeks postimmunization for reactivity to FEA as described in Example 10. As with immunization with unmodified gp70R-delta shown in Example 10, immunization with alkylated gp70R-delta elicits an anti-FeLV viral response by four weeks post-immunization. HPLC-purified adjuvants QA-7, QA-17, QA-18 all increase the immune response as compared to immunization in the absence of the saponin adjuvants. QA-17 and mixtures of QA-17 and QA-18 induced the highest response, inducing endpoint titers almost two orders of magnitude greater than immunization in the absence of saponin adjuvants. The results of these experiments are summarized on FIG. 14.

EXAMPLE 14

Toxicity of QA-7, QA-17, QA-18, QA-19, QA-21, "Quil-A"

With crude Quillaja saponins, a major symptom of toxicity in mice appears as necrosis of the liver. Purified saponins were injected into mice to determine effects on the liver. Mice were injected intradermally with 150 μg each QA-7, QA-17, QA-18, QA-21 and "Quil-A", the crude saponin extract used as the raw material for the purification of the other components. Animals injected with QA-7, QA-17, QA-18, and QA-21 appeared mildly ill initially but appeared to recover fully within a few hours after injection. "Quil-A" caused severe symptoms which continued for 48 hours. All mice were sacrificed at 48 hours for post-mortem examination of the liver. "Quil-A" caused severe damage of the liver with multifocal areas of acute necrosis evident. QA-7, QA-17, QA-18, and QA-21 did not seem to significantly affect the liver. QA-17 and QA-18 were also tested in kittens with subcutaneous injection of 100 μg each at 8 and 10 weeks, with no toxicity observed clinically or in the blood chemistry. In contrast, "Quil-A" induced a pyrogenic response which persisted for several hours in kittens. Hence, the purified saponins appear to be less toxic than "Quil-A" in both mice and kittens indicating that the purification process separates these saponins from one or more toxic components present in a crude Quillaja extract. One such toxic component has tentatively been identified as QA-19; dosages of 50 μg or greater were lethal in mice within a few days of injection. Further purification of QA-19 indicated that it could be separated into two peaks, QA-19a and QA-19b. QA-19a was lethal in mice at doses of 100 μg or greater whereas QA-19b was apparently nonlethal up to dose of 150 μg; hence, a synergistic effect to produce increased toxicity in the mixture of QA-19a and QA-19b cannot be ruled out. Preliminary screening of other minor peaks isolated from "Quil-A" indicates that other fractions may also be toxic. Hence, the purification protocols allow the separation of adjuvant-active saponins from similar but distinct compounds which are more toxic or which cochromatograph with toxic contaminants.

EXAMPLE 15

QA-18H and QA-21H, prepared as described in Example 7, were tested for adjuvant effect with BSA in direct comparison with the unhydrolyzed original products QA-18 and QA-21 prepared as described in Examples 3 and 4. QA-18 and QA-21 increase the humoral immune response to BSA in mice by at least an order of magnitude by two weeks post-immunization. However, the hydrolysis products QA-18H and QA-21H at the same weight dosage do not increase the response significantly (FIG. 15). Hence, optimal adjuvant effect is observed with the intact saponins; the essential structure required for adjuvant activity is lost or altered when QA-18 and QA-21 are hydrolyzed to QA-18H and QA-21H, respectively.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the invention as set forth below.

What is new and intended to be covered by Letters Patent of the United States is:

1. Substantially pure saponin purified from a crude *Quillaja saponaria* extract wherein said pure saponin is characterized by a single predominant peak which comprises 90% or more of the total area of all peaks of a chromatogram, excluding the solvent peak, when analyzed on reverse phase-HPLC on a Vydac C_4 column having 5 μm particle size, 330 Å pore, 4.6 mm ID×25 cm L in a solvent of 40 mM acetic acid in methanol/water (58/42; v/v) at a flow rate of 1 ml/mixture, and wherein said saponin has immune adjuvant activity and is less toxic when used as an adjuvant than said *Quillaja saponaria* extract.

2. Substantially pure QA-7 saponin purified from a crude *Quillaja saponaria* extract wherein said pure saponin is characterized by one predominant peak which comprises 90% or more of the total area of all peaks of a chromatogram, excluding the solvent peak, and having a retention time of approximately 9–10 minutes when analyzed on reverse phase HPLC on a Vydac C_4 column having 5 μm particle size, 330 Å pore, 4.6 mm ID×25 cm L in a solvent of 40 mM acetic acid in methanol/water (58/42; v/v) at a flow rate of 1 ml/minute.

3. The substantially pure QA-7 saponin of claim 2, wherein said saponin has immune adjuvant activity, and wherein said saponin is characterized by a carbohydrate content of about 35% per dry weight as assayed by anthrone, has a UV adsorption maxima of 205–210 nm, has a micellar concentration of 0.06% (w/v) in water and 0.07% in phosphate buffered saline, and causes no detectable hemolysis of sheep red blood cells at concentrations of 200 μg/ml.

4. The substantially pure QA-7 saponin of claim 3, wherein said carbohydrate content has a composition comprising the monosuccharides: terminal rhamnose, terminal xylose, terminal glucose, terminal galactose, 3-xylose, 3,4-rhamnose, 2,3-fucose, 2,3-glucuronic acid and apiose.

5. Substantially pure QA-21 saponin purified from a crude *Quillaja saponaria* extract wherein said pure saponin is characterized by one predominant peak which comprises 90% or more of the total area of all peaks of a chromatogram, excluding the solvent peak, and having a retention time of approximately 51minutes when analyzed on reverse phase-HPLC on a Vydac C_4 column having 5 μm particle size, 330 Å pore, 4.6 mm ID×25 cm L in a solvent of 40 mM acetic acid in methanol/water (58/42; v/v) at a flow rate of 1 ml/minute.

6. The substantially pure QA-21 saponin of claim 5, wherein said saponin has immune adjuvant activity, and wherein said saponin is characterized by a carbohydrate content of about 22% per dry weight as assayed by anthrone, has a UV absorption maxima of 205–210 nm, has a micellar concentration of about 0.03% (w/v) in water and 0.02% (w/v) in phosphate buffered saline, and causes hemolysis of sheep red blood cells at concentrations of 25 μg/ml or greater.

7. The substantially pure QA-21 saponin of claim 6, wherein said carbohydrate content has a composition comprising the monosaccharides: terminal rhamnose, terminal arabinose, terminal apiose, terminal xylose, 4-rhamnose, terminal glucose, terminal galactose, 2-fucose, 3-xylose, 3,4-rhamnose and 2,3-glucuronic acid.

8. A substantially pure QA-17 saponin purified from a crude *Quillaja saponaria* extract wherein said pure saponin is characterized by one predominant peak which comprises 90% or more of the total area of all peaks of a chromatogram, excluding the solvent peak, and having a retention time of approximately 35 minutes on reverse phase-HPLC on a Vydac C_4 column having 5 μm particle size, 330 Å pore, 4.6 mm ID×25 cm L in a solvent of 40 mN acetic acid in methanol/water (58/42; v/v) at a flow rate of 1 ml/minute.

9. The substantially QA-17 saponin of claim 8, wherein said saponin has immune adjuvant activity, and wherein said saponin is characterized by a carbohydrate content of about 29% per dry weight as assayed by anthrone, has a UV absorption maxima of 205–210 nm, has a micellar concentration of about 0.06% (w/v) in water and 0.03% (w/v) in phosphate-bufferred saline, and causes hemolysis of sheep red blood cells at concentrations of 25 μg/ml.

10. The substantially pure QA-17 saponin of claim 9, wherein said carbohydrate content has a composition comprising the monosaccharides: terminal rhamnose, terminal xylose, 2-fucose, 3-xylose, 3,4-rhamnose, 2,3-glucuronic acid, terminal glucose, 2-arabinose, terminal galactose and apiose.

11. A substantially pure QA-18 saponin purified from a crude *Quillaja saponaria* extract wherein said pure saponin is characterized by one predominant peak which comprises 90% or more of the total area of all peaks of a chromatogram, excluding the solvent peak, and having a retention time of approximately 38 minutes on reverse phase-HPLC on a Vydac C_4 column having 5 μm particle size, 330 Å pore, 4.6 mm ID×25 cm L in a solvent of 40 mM acetic acid in methanol/water (58/42; v/v) at a flow rate of 1 ml/minute.

12. The substantially QA-18 saponin of claim 11, wherein said saponin has immune adjuvant activity, and wherein said saponin is characterized by a carbohydrate content of about 25–26% per dry weight as assayed by anthrone, has a UV absorption maxima of 205–210 nm, has a micellar concentration of 0.04% (w/v) in water and 0.02% (w/v) in phosphate-buffered saline, and causes hemolysis of sheep red blood cells at concentrations of 25 μg/ml.

13. The substantially pure QA-18 saponin of claim 12, wherein said carbohydrate content has a composition comprising the monosaccharides: terminal rhamnose, terminal arabinose, terminal apiose, terminal xylose, terminal glucose, terminal galatose, 2-fucose, 3-xylose, 3,4-rhamnose and 2,3-glucuronic acid.

14. A method of enhancing an immune response to an antigen in an individual comprising administration of an amount of the substantially pure saponin adjuvants from any of claims 1–7 and 8–13 to said individual in an amount sufficient to enhance the immune response of said individual to said antigen.

15. A pharmaceutical composition useful for inducing the production of antibodies to an antigen in an individual comprising an immunogenically effective amount of an antigen and at least one substantially pure saponin as in any one of claims 1–7 and 8–13, wherein said substantially pure saponin is present in an amount sufficient to enhance the immune response of said individual to said antigen.

16. The pharmaceutical composition of claim 15, wherein said individual is a mammal.

* * * * *

Exhibit 3

United States Patent [19]

Myers et al.

[11] Patent Number: 4,912,094

[45] Date of Patent: Mar. 27, 1990

[54] **MODIFIED LIPOPOLYSACCHARIDES AND PROCESS OF PREPARATION**

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[73] Assignee: **Ribi ImmunoChem Research, Inc.**, Hamilton, Mont.

[21] Appl. No.: **212,919**

[22] Filed: **Jun. 29, 1988**

[51] **Int. Cl.**[4] **C07H 1/00**; C07H 13/02; C07G 17/00; C12P 19/04

[52] **U.S. Cl.** **514/54**; 536/124; 536/1.1; 536/119; 536/117; 536/115; 435/101

[58] **Field of Search** 536/124, 1.1, 119, 115, 536/117; 435/101; 514/54

[56] **References Cited**

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3,089,821	5/1963	Folkers	514/54
4,029,762	6/1977	Galanos et al.	530/387
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Primary Examiner—Ronald W. Griffin
Assistant Examiner—Everett White
Attorney, Agent, or Firm—Burgess, Ryan and Wayne

[57] **ABSTRACT**

Modified lipopolysaccharides, particularly de-3-0-acylated monophosphoryl lipid A and de-3-0-acylated diphosphoryl lipid A, are provided by an alkaline hydrolysis under controlled conditions which removes only the β-hydroxymyristic acyl residue that is ester-linked to the reducing-end glucosamine at position 3. The modified products are less endotoxic and maintain their antigenic and immuno-stimulating properties.

26 Claims, No Drawings

MODIFIED LIPOPOLYSACCHARIDES AND PROCESS OF PREPARATION

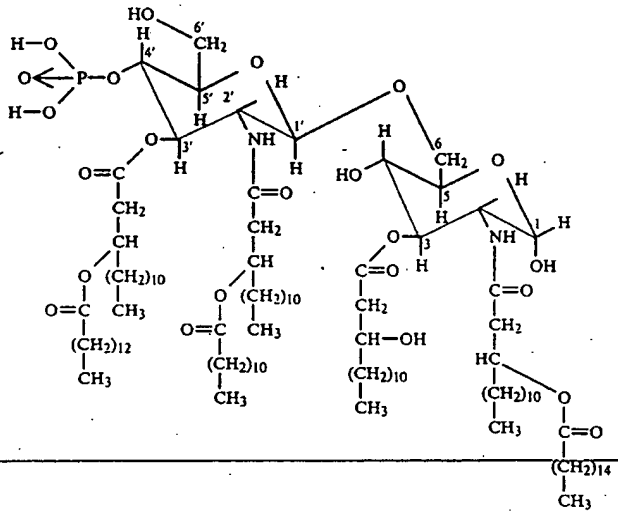
FIELD OF THE INVENTION

This invention relates in general to certain modified forms of lipopolysaccharide and lipid A. In one aspect, this invention is directed to a process for the structural modification of certain lipopolysaccharides to render them less endotoxic without adversely affecting their antigenic or immuno-stimulating properties.

BACKGROUND OF THE INVENTION

Prior to the present invention, it had long been recognized that enterobacterial lipopolysaccharides (LPS) was a highly potent stimulator of the immune system. A variety of responses, both beneficial and harmful, can be elicited by sub-microgram amounts of this substance. The fact that some of these responses are harmful, and can in fact be fatal, has to date precluded clinical use of LPS per se. It is now also well-appreciated that the endotoxic activites associated with bacterial lipopolysaccharides (LPS) reside in the lipid A component of LPS.

Accordingly, much effort has been expended towards attenuating the toxic attributes of lipid A and LPS without diminishing their beneficial immunostimulatory activities. Notable among these efforts was that of Edgar Ribi and his associates, which resulted in the production of a derivative of lipid A referred to originally as refined detoxified endotoxin (RDE) but more recently as monophosphoryl lipid A (MPL). MPL is produced by refluxing LPS (or lipid A) obtained from heptoseless mutants of gram negative bacteria (e.g. Salmonella sp.) in mineral acid solutions of moderate strength (e.g., 0.1N HCl) for a period of approximately 30 minutes. This treatment results in the loss of the phosphate moiety at position 1 of the reducing-end glucosamine. Coincidentally, the core carbohydrate is removed from the 6' position of the non-reducing glucosamine during this treatment. The result is the monophosphoryl derivative of lipid A, MPL. The structure of MPL is shown below:



(I)

MPL exhibits considerably attenuated levels of the endotoxic activities normally associated with lipid A and LPS, such as pyrogenicity, local Shwarzman reactivity, and toxicity in the chick embryo 50% lethal dose assay ($CELD_{50}$). It retains the ability of lipid A and LPS, however, and to, among other things, act as an adjuvant.

The difficulty with this method of detoxifying LPS and lipid A is that it invariably results in the loss of the core moiety attached to position 6' of the non-reducing glucosamine. This is significant since the core region is highly conserved among LPS's obtained from different genera of Enterobacteriaciae; immunity against the core region is therefore protective against a wide variety of gram negative bacterial challenges. This was demonstrated by the work of Ziegler et al. (New Eng. J. Med. 307, 1225: 1982), for example.

Considerable benefits would accrue from being able to immunize individuals against enterobacterial LPS, as evidenced by the fact that approximately 90,000 deaths occur annually from gram negative sepsis and associated endotoxemia. At the present time, however, it is only possible to immunize with fully toxid LPS, since detoxification by acid hydrolysis results in loss of the core region.

Alkaline hydrolysis has also been used in the past to detoxify LPS, but the conditions which have generally been used result in complete saponification of the lipid A moiety. This, of course, not only reduces the endotoxicity of the starting LPS, but also eliminates the other, more beneficial, activities as well. Furthermore, such treatment also reduces the immunogenicity of LPS, since it is essentially converted by this treatment into a polysaccharide antigen with no amphipathic character. In general, however, none of the early references teach that removal of one particular fatty acid from lipid A would render it non-toxic, while not affecting its immunostimulating activities.

Accordingly, one or more of the following objects will be achieved by the practice of this invention. It is an object of this invention to provide modified lipopolysaccharrides and, in particular, modified lipid A. Another object of this invention is to provide a modified lipid A which retains the core moiety attached to the 6' positions of non-reducing glucosamine. A further object

of the present invention is to provide a modified lipid A which retains the core moiety and accordingly, protection against a wide variety of gram negative challenges. A still further object of the present invention is to provide a process for the preparation of the modified lipid A which renders it less endotoxic without adversely affecting their antigenic or immunostimulating properties. Another object is to provide pharmaceutical compositions containing the modified lipopolysaccharides and a method for their use. These and other objects will readily become apparent to those skilled in the art in light of the teachings herein set forth.

SUMMARY OF THE INVENTION

In its broad aspect, the present invention is directed to certain modified forms of lipopolysaccharide and lipid A, and to a process for their preparation. The invention also encompasses pharmaceutical compositions containing the modified lipopolysaccharides and their use in the treatment of various conditions in warm blooded animals.

The modified lipopolysaccharides and lipid A of this invention are those which have been subjected to a mild alkaline hydrolysis under conditions as hereinafter defined that result in the loss of a single fatty acid from position 3 of the lipid A backbone.

DETAILED DESCRIPTION OF THE INVENTION

There is a considerable body of literature which pertains to the effect of alkaline treatment on the biological activities of LPS and lipid A. Most of these references teach the use of conditions that are sufficient to completely deacylate lipid A. As stated earlier, such treatment destroys essentially all biological activity of lipid A and LPS, except for antigenicity. The early paper by Neter et al. (Neter E., Westpahl O., Luderitz O., Gorzynski E. A. and Eichenberger E., "Studies of enterobacterial lipopolysaccharides". Effects of heat and chemicals on erythrocyte modifying, antigenic, toxic, and pyrogenic properties", J. Immunol. 76, 377: 1956), can be regarded as representative of the state of the art which teach the use of alkaline conditions sufficient to destroy all biological activities of LPS.

Several other observations have been noted in the scientific literature concerning alkaline hydrolysis of lipid A and LPS. For example, Niwa et al. (J. Bacterial. 97, 1069: 1969;) observed that treatment of LPS with mildly alkaline conditions caused a rapid loss of endotoxic activity and a much slower loss of fatty acids. This observation led them to conclude that the fatty acid-containing portion of LPS, lipid A, was not responsible for the endotoxic activity of LPS, since it was evidently destroyed at a slower rate than the endotoxic activity was lost. The authors conjectured that the only way that their observations might be consistent with lipid A being the endotoxic principle was if there existed a fatty acid in lipid A that was both highly alkaline-labile and necessary for endotoxic activity. The authors did not consider this to be a likely explanation. At the time, Niwa et al. favored an explanation for their results based on the influence of mild alkaline treatment on the conformation of endotoxin aggregates.

In a paper by Rietschel et al. (Eur. J. Biochem. 28; 166; 1973), it was noted that β-hydroxymyristic acid is rapidly released from lipid A upon mild alkali treatment (0.25N NaOH, 56° C.). The reason for the rapid loss of β-hydroxymyristic acid was not given, nor was it known from which position this fatty acid was cleaved. Also, no mention was made of the relationship of this rapid loss of β-hydroxymyristic acid to the loss of endotoxicity upon mild alkaline treatment which was observed by Niwa et al. and others.

In a paper by Goodman and Sultzer (Infect. Immunity 17, 205: 1977;) the authors noted that mild alkaline hydrolysis of LPS reduced its toxicity while actually enhancing its mitogenicity. They chemically characterized the hydrolyzed product with respect to nitrogen, glucosamine, KDO, and fatty acid content. Significantly, they found that the fatty acid content was relatively unchanged by the alkaline treatment. This led Goodman and Sultzer to conclude that the effect of the mild alkaline treatment was mediated by changes in the aggregational properties of the hydrolyzed LPS. In this regard, they were adopting the view of Niwa et al. On p. 212 of their paper, Goodman and Sultzer state that " . . . we have reduced the toxicity of the [LPS] by about 100-fold without significantly changing the lipid moiety." This confirms that they did not understand what they had done to achieve the observed reduction in toxicity without reducing mitogenicity. No mention was made of the possibility of a critical fatty acid that is alkaline-labile.

The sensitivity of ester-linked β-hydroxymyristic fatty acid residues present in lipid A to alkaline hydrolysis was noted in a 1982 publication (N. Qureshi, D. Takayama, and E. Ribi, J. Biol. Chem. 257, 11808: 1982). Similar observations were made with respect to a monosaccharide precursor of lipid A in a 1983 publication (Takayama, et al., J. Biol. Chem 258, 14245: 1983). Both of these references teach that ester-linked β-hydroxymyristic fatty acid residues present in lipid A or related compounds are easily removed by mild alkaline treatment. The effect of this structural modification on the biological activity of lipid A was not recognized in this or any subsequent references.

The treatment of LPS with mild alkali was discussed in two papers by Amano, et al. (D. Amano, E. Ribi, and J. L. Cantrell, J. Biochem 93, 1391: 1983, and K. Amano, E. Ribi, and J. L. Cantrell, BBRC 106, 677: 1982). The authors reported that mild alkali treatment results in the loss of O-ester linked fatty acids. They did not mention that the only fatty acid removed by this treatment is the β-hydroxymyristic at position 3. Also, contrary to the results disclosed in the present invention, they reported that mild alkali treatment did not reduce the endotoxicity of the parent LPS.

A study of the structural consequences of treating LPS with mild alkali was reported in a paper by Rosner, et al. (M. R. Rosner, J-y Tang, I. Barzilay, and H. G. Khorana, J. Biol. Chem 254, 5906: 1979). The authors reported that LPS which was treated with 1N NaOH at room temperature for approximately 17 hrs was exhaustively de-O-acylated. This is clearly different from the present invention, which discloses conditions sufficient to remove only the β-hydroxymyristic from position 3. Furthermore, the authors subjected LPS to this mild alkali treatment solely for the purpose of elucidating LPS's structure. No mention is made in this article of the effect of mild alkali treatment on the biological activities of LPS.

The use of mild alkali treatment to lower the toxicity of lipid A was disclosed in U.S. Pat. No. 4,029,762. this patent discloses the use of lipid A and alkali-treated lipid A as antigens for stimulating immunity against gram-negative enterobaceriaceae. It was not disclosed

in this patent that lipid A, which lacked a β-hydroxymyristic acid at position 3, is less endotoxic but is still mitogenic.

In a 1987 review by Rietschel et al. (in "Detection of Bacterial Endotoxins with the Limulus Amebocyte Lysate Test", Alan R. Liss, Inc., 1987, p. 25–53;), mention is made of the fact that synthetic monosaccharides corresponding to the reducing end of lipid A are inactive if the β-hydroxymyristic acid residue at position 3 is removed. The authors, however, did not conjecture as to whether the same observation would be made with lipid A, and no work was cited pertaining to this question. It does not appear then that it was suspected that removal of the β-hydroxymyristic acid from position 3 of lipid A and LPS would result in reduced endotoxicity without affecting activities such as mitogenicity.

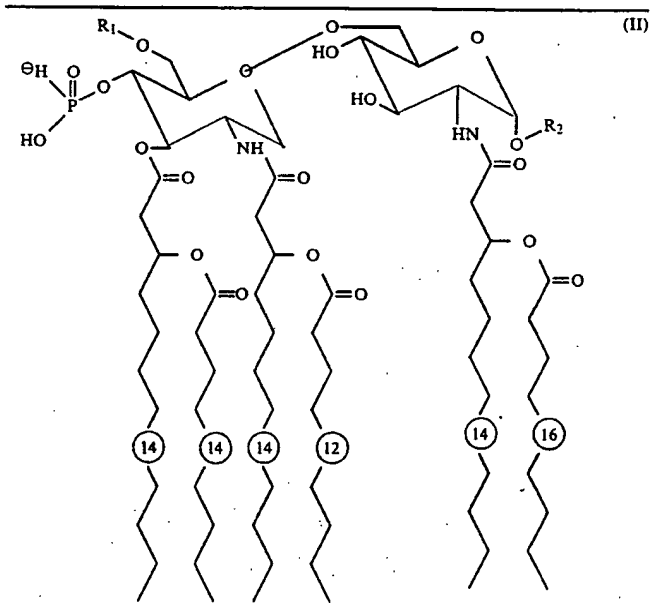
Accordingly, prior to the present invention, and in view of the reported research efforts of Edgar Ribi and his colleagues, in preparing and evaluating monophosphoryl lipid A, it was generally recognized that detoxification of lipopolysaccharide was best accomplished by an acid hydrolysis followed by a chromatographic separation of MPL if a product having enhanced immuno-stimulating properties was desired. It was not readily apparent that the endotoxicity of lipid A could be attenuated by removal of only the fatty acid at position 3 or that removal of the position 3 fatty acid from lipopolysaccharide would reduce endotoxicity and yet allow other desirable substituents to remain in the molecule.

Thus, in contrast to the prior art references which may disclose, in general, the alkaline treatment of lipopolysaccharides, none of these references clearly recognizes the unexpected and surprising results obtained by employing conditions for removal of only the β-hydroxymyristic acyl residue from lipopolysaccharides and thereafter separating and recovering the deacylated product in a relatively pure form.

Lipid A deacylated in accordance with the method of the present invention was found to be non-toxic in the $CELD_{50}$ assay ($CELD_{50} > 10$ μg), in spite of the fact that it still contained (1) a diglucosamine backbone, (2) two phosphoryl groups, (3) at least two 3-acyloxyacyl residues, and (4) up to a total of 6 fatty acids. Taken together, these results indicate that the total number of fatty acids present in lipid A is not a sufficient condition for the manifestation of endotoxic activity, but that the pattern of fatty acid substitution is also a critical determinant.

While not wishing to be bound by any theory regarding the reasons why the compound(s), although less endotoxic, are still able to exert a strong immunostimulating effect, it is believed that the specific structural modification that is responsible for this reduction in the endotoxicity of lipid A and LPS involves removing of the β-hydroxymyristic acyl residue that is ester-linked to the reducing-end glucosamine at position 3 under conditions which do not adversely affect other groups in the lipopolysaccharides. Monophosphoryl lipid A (MPL), diphosphoryl lipid A (DPL) and LPS can all be de-3-O-acylated in this way. The structures of these novel materials are shown below in formula II wherein the figures in the circles indicates the number of carbon atoms in the chain.

(II)



Compound	R1	R2
d3-MPL	H	H
d3-DPL	H	PO_3H^-
d3-LPS	Core (±O-antigen)	PO_3H^-

Various forms of de-3-O-acylated materials are encompassed by this invention. The lipid A backbone that is shown corresponds to the product that is obtained by de-3-O-acylation of heptaacyl lipid A from *S. minnesota* R595. Other fatty acid substitution patterns are encompassed by this disclosure; the essential feature is that the material be de-3-O-acylated.

Thus, one embodiment of this invention is directed to the composition of MPL, DPL and LPS in which the position 3 of the reducing end glucosamine is de-O-acylated. These compounds as indicated above are referred to as d3-MPL, d3-DPL, and d3-LPS, respectively.

Also as indicated above, the modified lipopolysaccharides of the present invention are prepared by subjecting the compounds to alkaline hydrolysis under conditions that result in the loss of but a single fatty acid from position 3 of the lipid A backbone.

The β-hydroxymyristic at position 3 is unusually labile in alkaline media. It requires only very mild alkaline treatment to completely de-3-O-acylate lipid A and LPS. The other ester linkages in lipid A and LPS require somewhat stronger conditions before hydrolysis will occur, so that it is possible to selectively deacylate these materials at position 3 without significantly affecting the rest of the molecule. The reason for the unusual sensitivity to alkaline media of the ester-linked β-hydroxymyristic at position 3 is not known at this time.

Although alkaline hydrolysis procedures are known, it is important to choose conditions that do not cause further hydrolysis beyond the ester linkage to the β-hydroxymyristic at position 3.

In general, the hydrolysis can be carried out in aqueous or organic media. In the latter case, solvents include methanol (alcohols), dimethyl sulfoxide (DMSO), dimethylformanide (DMF), chloroform, dichloromethane, and the like as well as mixtures thereof. Combinations of water and one or more of these organic solvents also can be employed.

The alkaline base can be chosen from among various hydroxides, carbonates, phosphates and amines. Illustrative bases include the inorganic bases such as sodium hydroxide, potassium hydroxide, sodium carbonate, potassium carbonate, sodium bicarbonate, potassium bicarbonate, and the like, and organic bases such alkyl amines and include, but are not limited to, diethylamine, triethylamine and the like.

In aqueous media, the pH is typically between approximately 10 and 14 with a pH of about 12 to about 13.5 being the preferred range. The hydrolysis reaction is typically carried out at a temperature of from about 20° to about 80° C., preferably about 50° to about 60° C. for a period of about 10 to about 30 min. For example, the hydrolysis can be conducted in 3% triethylamine in water at room temperature (22°–25° C.) for a period of 48 hrs. The only requirement in the choice of temperature and time of hydrolysis is that de-O-acylation occurs to remove only the β-hydroxymyristic at position 3.

In practice, it has been found that a particularly desirable hydrolysis method involves dissolving liquid A or monophosphoryl lipid A in chloroform:methanol 2:1 (v/v), saturating this solution with an aqueous buffer consisting of 0.5M $Na_2CO)_3$ at pH 10.5, and then to flash evaporate the solvent at 45°–50° C. under a vacuum for an aspirator (approximately 100 mm Hg). The resulting material is selectively deacylated at position 3. This process can also be carried out with any of the inorganic bases listed above. The addition of a phase transfer catalyst, such as tetrabutyl ammonium bromide, to the organic solution prior to saturating with the aqueous buffer may be desirable in some cases.

In preparing the modified lipopolysaccharides of this invention, it is deemed highly important that LPS can be deacylated at position 3 without causing any changes in the O-antigen or core regions or in the structure of the lipid A component except for loss of the labile fatty acyl residue. There are several implications of this result with respect to possible uses of the de-3-O-acylated compounds. For example, vaccines against gram negative bacteria and/or endotoxin can be generated using LPS that has been treated in the manner of this disclosure which results in a preparation with low endotoxicity but with the same antigenic attributes as the parent material, and which is able to act as its own adjuvant. Such preparations may be able to promote a strong specific immune response without the toxic effects generally associated with LPS-based vaccines.

Another implication is that lipid A that has been detoxified by de-3-O-acylation, since it still contains both phosphates, may have greater immunostimulatory activities than lipid A that has been detoxified by the prior art method involving acid hydrolysis to remove the reducing end phosphate. For example, acid hydrolyzed LPS, which is referred to as monophosphoryl lipid A (MPL), is less mitogenic with respect to B-lymphocyte proliferation than is d3-LPS. Thus, de-3-O-acylated lipid A and LPS may be more potent immunostimulators than is MPL. Furthermore, because of structural differences between MPL and de-3-O-acylated lipid A and LPS, the latter compounds may exhibit a different spectrum of beneficial biological activities than does MPL.

It is therefore viewed as a significant advance to be able to reduce the endotoxicity of LPS without eliminating its antigenic attributes or its immunostimulating activity. LPS subjected to mild alkaline hydrolysis can be used to immunize warm blooded animals including humans, thus conferring protection against gram-negative septicemia and associated endotoxemia.

A further advantage of d3-LPS and d3-DPL relative to MPL is that these materials, since they possess both of the phosphate groups present in lipid A, may exhibit enhanced activities relative to MPL, which is lacking the phosphate moiety at the 1 position. This has already been found with respect to mitogenicity; d3-LPS is as mitogenic as the parent LPS, whereas MPL is only about half as mitogenic.

Finally, the conditions used to effect the mild alkaline hydrolysis disclosed herein are, in some cases, easier to attain than those of the prior art methods for detoxifying LPS or lipid A. For example, as mentioned above, lipid A can be detoxified by dissolving it in a solution chloroform:methanol 2:1 (v/v), saturating this solution with an aqueous buffer consisting of 0.5M Na_2CO_3 at pH 10.5, and then evaporating the solvent at 45°–50° C. This method is also effective in removing the residual endotoxicity which is usually found in crude preparations of MPL, and which is typically removed by chromatographic purification. Thus, milk alkaline hydrolysis can obviate the need for the costly and time-consuming chromatography steps which are generally required in order to fully-detoxify preparations of MPL.

The lipopolysaccharide which is free of the β-hydroxymyristic acid residue, can be covered from the reaction medium in relatively pure form.

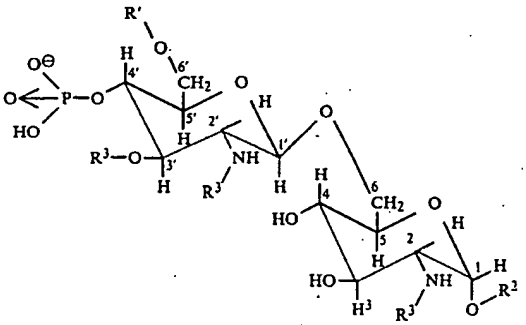
Although the present invention is particularly useful for de-3-O-acylating MPL, it is applicable to lipopolysaccharides in general. Lipopolysaccharides represent a biologically-active-class-of-substances-and-chemically are made up of a polysaccharide portion, the O-specific chains (O-antigen) and the core, and a covalently bound lipid, lipid A. Lipid A represents the endotoxically active region of lipopolysaccharides, while the polar

polysaccharide part serves as a solubilizing carrier. Lipid A of Salmonella consists of a backbone of β-1,6-linked D-glucosamine disaccharide units which are substituted at positions 1 and 4' by phosphate-residues and at position 6' by the core polysaccharide. The other hydroxyl and the amino groups of the backbone are acylated by long-chain fatty acids, of which lauric, myristic, palmitic and 3-hydroxymyristic acid predominate.

The term "monophosphoryl lipid A", "(MPL)" or "(MLA)" as used herein is meant to designate the monophosphoryl lipid A of structure I and is obtained from lipopolysaccharides such as *Salmonella minnesota* R 595, *Escherichia coli*, and the like. MLA is reported by N. Qureshi et al. Journal of Biological Chemistry, Vol 260, No. 9. pages 5271–5278 (1985).

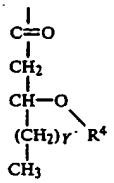
Accordingly, the lipopolysaccharide compounds of this invention after de-3-O-acylation, can also be represented by the formula.

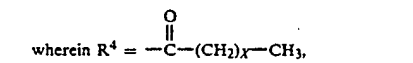
A lipopolysaccharide compound of the formula



wherein R^1 is selected from the group consisting of hydrogen and the core component of enterobacterial lipopolysaccharide, with or without the O-antigen present, R^2 is selected from the groups H and PO_3H_2, and a R^3 is selected from the group consisting of H, β-hydroxymyristoyl, and a 3-acyloxyacyl residue having the formula:

III





and wherein X and Y can have a value of from 0 up to about 20 and preferably 10, 12 or 14.

The compounds prepared by the present invention are therefore substantially pure compounds and not mixtures of partially de-O-acylated compounds.

All of the uses that are disclosed in the literature for MPL can be entertained with respect to d3-MPL and, especially, d3-DPL. These include (a) use as an adjuvant, (b) protection against radiation, (c) protection against gram negative septicemia and associated endotoxemia, (d) protection against non-specific infectious challenges, and (e) treatment of neoplastic disease. The de-3-O-acylated materials are used in the same way as MPL in all of these applications, i.e., at the same doses, in the same combinations.

Additionally, d3-LPS can be used as a vaccine against gram-negative infections. In this regard, the material is prepared from LPS obtained from either a wild-type strain of a gram negative organism or else from a strain that has a partially-complete (and therefore antigenically cross-reactive) core region (e.g. *E. coli* J5). Such d3-LPS can be administered either in saline, in a lipid emulsion system, or in an oil-in-water emulsion (1–2% squalane or squalene, 0.2% Tween 80). In the latter case, other bacterially-derived immunostimulants (CWS, TDM) can be used in combination with D3-LPS. The amount of d3-LPS per dose is between about 10 and about 1000 μg, and preferably between about 20 and about 200 μg. CWS and TDM, if used, and at similar levels per dose.

The following examples are illustrative of the present invention.

EXAMPLE 1

Removal of the β-Hydroxymyristic Acid at Position 3 of *Escherichia coli* D31M4 MPL by Treatment with Organic Alkaline Media

580 mg of crude *E. coli* D31m4 MPL was dissolved in 250 mls of chloroform:methanol 2:1 (v/v). This solution was transferred to a 1 liter separatory funnel, where it was washed with 100 mls 0.5M Na_2CO_3, pH 10.5. The organic phase was removed, and the solvent was stripped off by flash evaporation using a water aspirator and a bath temperature of 45° C. The resulting residue contained 615.9 mg of de-3-O-acylated MPL (d3-MPL), as judged by thin layer chromatography (Silica gel 60, chloroform:methanol:water:ammonium hydroxide 50:31:6:2 (v/v); plates visualized by spraying with ammonium molybdate in ethanol (10% w/v) and charring).

EXAMPLE 2

The 50% Lethal Dose in Chick Embryos ($CELD_{50}$ of Crude MPL Before and After De-3-O-Acylation

MPL and d3-MPL (prepared in Example 1, above) were dispersed in sterile water containing 0.2% triethylamine (TEA; v/v) to a concentration of 2.0 mg/ml. An ultrasonic bath and mild warming (45°–50° C.) promoted solubilization. To these solutions were added equal volumes of 1.8% NaCl (w/v), giving final solutions that contained 1.0 ug/ml MPL or d3-MPL, 0.9% NaCl (w/v), and 0.1% TEA (v/v). The toxicity of these solutions in 11 day-old chick embryos was then assessed by the method of Milner and Finklestein (J. Infect. Diseases 116, 259: 1966). The chick embryo 50% lethal doses ($CELD_{50}$) were calculated by the method of Reed and Muench (Am. J. Hyg. 27, 493: 1938). The $CELD_{50}$ of the crude MPL used in Example 1 was found to be less than 1 μg. On the other hand, the d3-MPL did not kill any chick embryos even at 20 μg, the highest dilution tested.

EXAMPLE 3

De-3-O-Acylation-of-*Salmonella-minnesota*-R595-LPS by Treatment with Aqueous TEA

Into a 4 ml screw-top vial was placed 10.1 mg *S. minnesota* R595 LPS. 2.0 ml sterile water was added to the vial, which was then capped and sonicated for 3

min. at room temperature. The vial was then placed in a boiling water bath. After 5 min. the vial was removed from the bath and 67 ul TEA was added to the solution, with stirring. The vial was capped and allowed to stand at room temperature for 43 hr. At this time, the extent of de-3-O-acylation was assessed by first subjecting a small portion of the reaction solution to acid hydrolysis, in order to convert all of the LPS to MPL. This was accomplished by adding 0.3 ml of 0.47N HCl to a 0.2 ml aliquot of the reaction solution, then placing the acidified solution into an oil bath (130° C.) for 10 min. The solution was stirred during this time. The solution was then cooled in an ice-water bath, and the MPL was extracted using 1.0 ml of chloroform:methanol 2:1 (CM 2:1; v/v). A control solution was prepared by dispersing 1.00 mg LPS in 0.2 ml water plus 6.7 ul TEA, adding 0.3 ml of 0.47N HCl, incubating in a 130° C. oil bath for 10 min., cooling and extracting with CM 2:1. The alkaline-hydrolyzed material and the control were then analyzed by TLC, as described in Example 1. TLC revealed that almost all of the MPL from the TEA-treated LPS was de-3-O-acylated, which indicated that the TEA treatment had resulted in the production of d3-LPS. The control sample, which had not been exposed TEA for the extended period, appeared identical to MPL from untreated LPS. The TEA hydrolysis reaction was therefore judged to be complete. The remaining reaction mixture was dialyzed against distilled water (6,000–8,000 MWt cutoff) and lyophilized, yielding 8.45 mg d3-LPS.

EXAMPLE 4

Biological Activity of d3-LPS

The endotoxicity of the d3-LPS prepared in example 3 was compared with that of the starting LPS using the $CELD_{50}$ assay, as described in Example 2. The activities of LPS and d3-LPS were also evaluated in a lymphocyte proliferation assay, based on uptake of ^3H-thymidine by murine spleen cells following exposure to these materials. The results from these assays are shown in Table 1. They indicate that d3-LPS, while much less endotoxic than the parent LPS, is still a potent mitogen.

TABLE 1

The effect of de-3-O-acylation on the biological activities of *S. minnesota* R595 LPS.

Sample	$CELD_{50}$[a]	Mitogenicity[b] C3H/FeJ[c]	Mitogenicity[b] CeH/HeJ[c]
LPS	0.03 μg	33.9	4.4
d3-LPS	1.4 μg	30.0	5.2

Notes:
[a]The dose necessary to cause 50% mortality in 11 day old chick embryos.
[b]Lymphocyte proliferation assay, based on uptake of ^3H-thymidine by murine spleen cells. The numbers represent the ratio of ^3H counts in stimulated cells to counts in unstimulated cells.
[c]C3H/FeJ mice are LPS-responsive; C3H/HeJ mice are LPS-unresponsive.

EXAMPLE 5

The Rate of De-3-O-Acylation and Detoxification of *S. minnesota* R595 Diphosphoryl Lipid A (DPL) in Organic Alkaline Media

Into each of 4 100×16 mm test tubes was placed 2.0 mg *S. minnesota* R595 DPL. To each tube was added 5 ml-CM-2:1-and-2-ml-0.5M-Na_2CO_3-pH-10.5. The test tubes were vortexed, centrifuged for 5 min at 3000 g, and the organic layers were withdrawn and transferred to clean test tubes. These solutions were then incubated for varying periods of time at 51°–52° C. (0, 2, 5, and 10 min). The reactions were quenched at the indicated times by placing the tubes in an ice-water bath and adding ice chips to the solutions. After about 30 sec, 2.0 ml of 0.1N HCl was added to each test tube, and the tubes were vortexed and centrifuged. The organic layers were transferred to clean test tubes and washed with distilled water (plus ice chips). Finally, the organic layers were evaporated under a stream of nitrogen. A 0.4 mg portion of each residue was subjected to acid hydrolysis by the method described in Example 3, in order to convert the residues to the corresponding MPLs. The MPLs were then analyzed by TLC as described in Example 1, and the endotoxicities of the corresponding DPL residues from each time point were measured with the $CELD_{50}$ assay, as described in Example 2. The results are summarized in Table II.

TABLE II

The Rate of De-3-O-Acylation and Detoxification of *S. minnesota* R595 Diphosphoryl Lipid A (DPL) in Organic Alkaline Media.

Incubation Time[a]	Extent of De-3-O-Acylation[b]	$CELD_{50}$[c]
0 min	None	0.085 μg
2	Half	NT
5	Almost complete	1.78
10	Complete	10 μg

Notes:
[a]The time each tube was incubated at 51–52° C.
[b]As judged visually from the TLC appearance of the MPL corresponding to the DPL at each time point.
[c]The dose necessary to cause 50% mortality in 11 day-old chick embryos.

Although the invention has been illustrated by the preceding examples, it is not to be construed as being limited to the materials employed therein but rather, the invention relates to the generic area as herein before disclosed. Various modifications and embodiments thereof can be made without departing from the spirit or scope thereof.

What is claimed is:

1. A method for modifying a lipopolysaccharide to selectively remove only the β-hydroxymyristic acyl residue that is ester-linked to the reducing-end glucosamine at position 3 of said lipopolysaccharide, which comprises subjecting said lipopolysaccharide to alkaline hydrolysis sufficient only to remove β-hydroxymyristic acid from position 3 without removal of other fatty acids from the lipopolysaccharide molecule and recovering said lipopolysaccharide free of said residue.

2. The method of claim 1 wherein said lipopolysaccharide is enterobacterial lipopolysaccharide.

3. The method of claim 1 wherein said lipopolysaccharide is monophosphoryl lipid A.

4. The method of claim 1 wherein said lipopolysaccharide is diphosphoryl lipid A.

5. The method of claim 1 wherein said hydrolysis is conducted in the presence of sodium carbonate.

6. The method of claim 1 wherein said hydrolysis is conducted in the presence of triethylamine.

7. The method of claim 1 wherein said hydrolysis is conducted in an organic medium.

8. The method of claim 1 wherein said hydrolysis is conducted in an aqueous medium.

9. The method of claim 1 wherein said hydrolysis is conducted at a pH of from about 10 to about 14 and at a temperature of from about 20° to about 80° C.

10. A method for removing from lipid A or monophosphoryl lipid A, only the β-hydroxymyristic acyl

residue that is ester-linked to the reducing-end glucosamine at position 3 of said lipid A, which method comprises the steps of:

(a) dissolving said lipid A in an inert organic solvent;

(b) saturating said solvent with an aqueous buffer comprised of an alkaline compound, at a pH of from about 10 to about 13;

(c) flash evaporating said solvent under a vacuum at a temperature of between about 40° C. and about 60° C.; and

(d) recovering said lipid A.

11. The method of claim **10** wherein said lipid A is monophosphoryl lipid A.

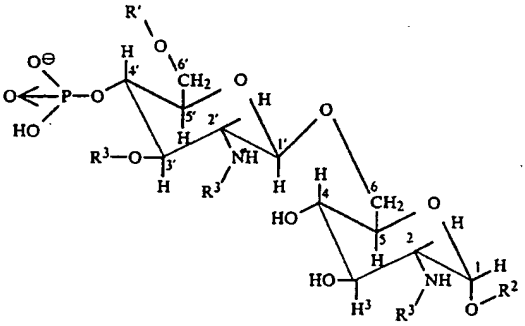
12. The method of claim **10** wherein said inert, organic solvent is a mixture of chloroform and methanol.

13. The method of claim **10** which is effected in the presence of a phase transfer catalyst.

14. The method of claim **13** wherein said phase transfer catalyst is tetrabutyl ammonium bromide.

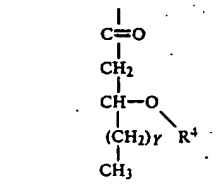
15. A lipopolysaccharide from which the β-hydroxymyristic acyl group has been removed from position 3.

16. An essentially pure lipopolysaccharide compound of the formula:



wherein R^1 is selected from the group consisting of hydrogen and the core component of enterobacterial lipopolysaccharide, with the O-antigen present, R^2 is selected from the groups H and PO_3H_2, and R^3 is selected from the group consisting of H, β-hydroxymyristoyl, and a 3-acyloxyacyl residue having the formula:

(III)



wherein $R^4 = -\overset{O}{\overset{\|}{C}}-(CH_2)_X-CH_3$,

and wherein X and Y have a value of from 0 up to about 20.

17. The compound of claim **16** wherein R^1 and R^2 are hydrogen.

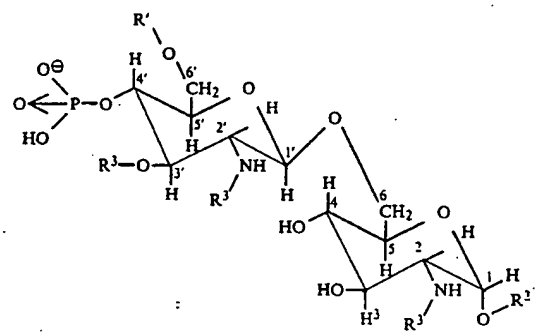
18. The compound of claim **16** wherein R^1 is hydrogen and R^2 is PO_3H_2.

19. The compound of claim **16** wherein R^1 is the core component of enterobacterial lipopolysaccharide and R^2 is PO_3H_2.

20. The compound of claim **16** wherein R^1 and R^2 are hydrogen, and R^3 is the residue of Formula III.

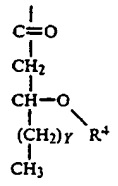
21. The compound of claim **20** wherein X has a value of 10, 12 or 14 and Y is 10.

22. An essentially pure lipopolysaccharide compound of the formula:



wherein R^1 is selected from the group consisting of hydrogen and the core component of enterobacterial lipopolysaccharide, without the O-antigen present, R^2 is selected from the groups H and PO_3H_2, and R^3 is selected from the group consisting of H, β-hydroxymyristoyl, and a 3-acyloxyacyl residue having the formula:

(III)



wherein $R^4 = -\overset{O}{\overset{\|}{C}}-(CH_2)_X-CH_3$,

and wherein X and Y have a value of from 0 up to about 20.

23. A pharmaceutical composition comprising a substantially pure, modified lipopolysaccharide, having no β-hydroxymyristic acyl residue that is ester-linked to the reducing-end glucosamine at position 3, and a pharmaceutically acceptable carrier.

24. The pharmaceutical composition of claim **23** wherein said lipopolysaccharide is enterobacterial lipopolysaccharide.

25. The pharmaceutical composition of claim **23** wherein said lipopolysaccharide is monophosphoryl lipid A.

26. The pharmaceutical composition of claim **23** wherein said lipopolysaccharide is diphosphoryl lipid A.

* * * * *

Exhibit 4

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CONTENTS

(continued on next page)

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Infection of Cynomolgus Monkeys with a Chimeric HIV-1/SIV_{mac} Virus That Expresses the HIV-1 Envelope Glycoproteins

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Summary: Replication competent chimeric viruses that express the gag and pol proteins of SIV_{mac} and the env proteins of HIV-1 were made. One such chimeric virus, SHIV-4, that expresses the *vif*, *vpx*, *vpr*, and *nef* regulatory genes of SIV and the *tat* and *rev* regulatory genes of HIV-1 replicated efficiently in cynomolgus monkeys. This model system can be used to evaluate the efficacy of anti-HIV-1 vaccines directed at the envelope glycoproteins, anti-HIV-1 envelope glycoprotein antiserum or monoclonal antibodies, and anti-HIV-1 drugs designed to inhibit the *tat*, *rev*, or *env* functions. Key Words: Chimeric virus—HIV/SIV—Cynomolgus monkey.

Human immunodeficiency virus type 1 (HIV-1) and human immunodeficiency virus type 2 (HIV-2) are the etiologic agents of AIDS in humans (1–3). These viruses are related to simian immunodeficiency viruses (SIV) that infect feral populations of sooty mangabeys, African green monkeys, and mandrills (for review, see refs. 4 and 5).

Development of a vaccine to prevent infection of HIV-1 requires a suitable animal model. The two animal models most commonly used, infection of chimpanzees with HIV-1 and infection of macaque monkeys with SIV, have limitations. HIV-1 does not replicate to high titers in chimpanzees, and infected chimpanzees do not develop immunodeficiency (6–10). Trials in chimpanzees are limited to a few animals as the species is endangered, available chimpanzees are few, and care is expensive.

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Rhesus and cynomolgus macaque monkeys infected with the macaque strain of SIV (SIV_{mac}) do produce high titers of virus and do develop an AIDS-like syndrome (11–13). Differences exist, however, in the immune responses to the HIV-1 and SIV_{mac} envelope glycoproteins, which represent the principal targets for protective immunity (14–16). The major neutralizing antibodies in HIV-1–infected people are directed against two regions of the gp120 envelope glycoprotein. Antibodies against the HIV-1 third gp120 variable (V3) region have been shown to be protective (15). In contrast, the corresponding region of the SIV_{mac} envelope glycoprotein does not exhibit sequence variation among isolates and is not a target for neutralizing antibodies in infected macaques (17,18). A second group of neutralizing antibodies in HIV-1–infected humans is directed against the conserved, discontinuous gp120 region that binds the CD4 viral receptor (19–29). These antibodies recognize HIV-1 gp120 regions distinct from those of the SIV_{mac} gp120 glycoprotein recognized by antibodies from

infected macaques that neutralize multiple SIV strains (30–33). The differences between the antibodies that broadly neutralize HIV-1 and SIV strains are highlighted by the observation that such antibodies do not cross-neutralize (34).

To overcome these difficulties an attempt was made to create a chimeric virus between HIV-1 and SIV_{mac} that contains the HIV-1 envelope glycoproteins and is capable of replicating to high titers in macaque monkeys.

MATERIALS AND METHODS

Plasmid Constructions

The chimeric viruses were constructed using the infectious, pathogenic $SIV_{mac}239$ (*nef* open) virus (*gag*+, *pro*+, *pol*+, *vif*+, *vpx*+, *vpr*+, *tat*+ *rev*+, *env*+, *nef*+) (12,35) and the HXBc2 HIV-1 virus (*gag*+, *pro*+, *pol*+, *vif*+, *vpr*−, *tat*+, *rev*+, *vpu*−, *env*+, *nef*−) (36). All four chimeric viruses (designated SHIV) used in this study express the gag, pro, pol, vif, vpr, and nef proteins of $SIV_{mac}239$ (*nef* open) and the tat, rev, and env proteins of HIV-1 (HXBc2).

Each chimeric provirus clone was propagated in *E. coli* using two plasmids, one containing the 5′ half of the provirus and one containing the 3′ half of the provirus. The 5′ proviral clones, derived from the p239 SpSp 5′ plasmid (12), consisted entirely of sequences from the $SIV_{mac}239$ clone. The sequences from the 5′ cellular flanking sequences to the unique *Sph* I site in the $SIV_{mac}239$ genome were cloned into a pBS(+) plasmid (Stratagene) modified to contain a unique *Cla* I site in the polylinker region. This 5′ clone, which was used to generate the SHIV-1 and SHIV-2 chimeric viruses, contains the $SIV_{mac}239$ *tat* splice acceptor and *tat* initiation codon. Site-directed mutagenesis was used to create a modified 5′ clone in which the $SIV_{mac}239$ *tat* splice acceptor and *tat* initiation codon were modified (Fig. 1) This modified 5′ clone was used to generate the SHIV-3 and SHIV-4 chimeric viruses.

The 3′ proviral clones consisted of *tat*, *rev* and *env* sequences derived from the HXBc2 HIV-1 isolate and the *nef* and 3′ LTR sequences derived from the $SIV_{mac}239$ (*nef* open) isolate (35). In the $SIV_{mac}239$ (*nef* open) variant, the 93rd codon of *nef* is changed from a stop (TAA) to a Glu (GAA) codon, allowing production of a functional *nef* protein (35). The HIV/SIV_{mac} junction in the 3′ proviral clones was formed by ligating the HIV-1 and SIV_{mac} segments using the *Rsr* II site, which was created by site-directed mutagenesis in both the HIV-1 and $SIV_{mac}239$ (*nef* open) sequences.

To allow efficient ligation of the 5′ and 3′ proviral halves, a unique *Sph* I site was introduced by site-directed mutagenesis into the HIV-1 region upstream of the HIV-1 *tat* gene. This *Sph* I site was positioned such that the HIV-1 *tat* splice acceptor sequences would be either included in or excluded from the 3′ proviral clones. The 3′ clone that included the HIV-1 *tat* splice acceptor was used to generate the SHIV-1 and SHIV-3 chimeric viruses, while the 3′ clone lacking the HIV-1 *tat* splice acceptor was used to generate the SHIV-2 and SHIV-4 viruses (Fig. 1). Also, since the last few codons of the SIV_{mac} *vpr* gene are located 3′ to the natural *Sph* I site in the 5′ proviral clone, these codons were supplied by modification of the 3′ proviral clone near the introduced *Sph* I site. Thus, the *vpr* reading frame would be restored upon ligation of the 5′ and 3′ proviral clones at the *Sph* I site (Fig. 1).

Transfection of CEMx174 Cells with Chimeric Proviruses

For transfection, 5 μg of the 5′ and 3′ proviral clones were digested with *Sph* I and other restriction enzymes that recognize the flanking sequences (*Cla* I for the 5′ proviral clone and *Xho* I for the 3′ proviral clone). The fragments containing the 5′ and 3′ proviral sequences were ligated. The ligation reaction was then mixed with 3×10^6 CEMX174 cells suspended in 1 ml of serum-free RPMI 1640 and 500 μg/ml DEAE–dextran. The cell–DNA suspension was incubated at 37°C for 1 h, after which the cells were washed with serum-free medium and resuspended in 10 ml RPMI 1640 with 10% fetal calf serum.

Reverse Transcriptase Assays

Virus production in transfected or infected cultures was monitored every 3–4 days by reverse transcriptase assays as described, using 1.5 ml of cell-free supernatant (37). After removing supernatants for reverse transcriptase assays, cells were resuspended in a sufficient amount of fresh medium to maintain the cell density between 10^5 and 10^6 cells/ml.

Infection of Cultured Monkey PBMCs

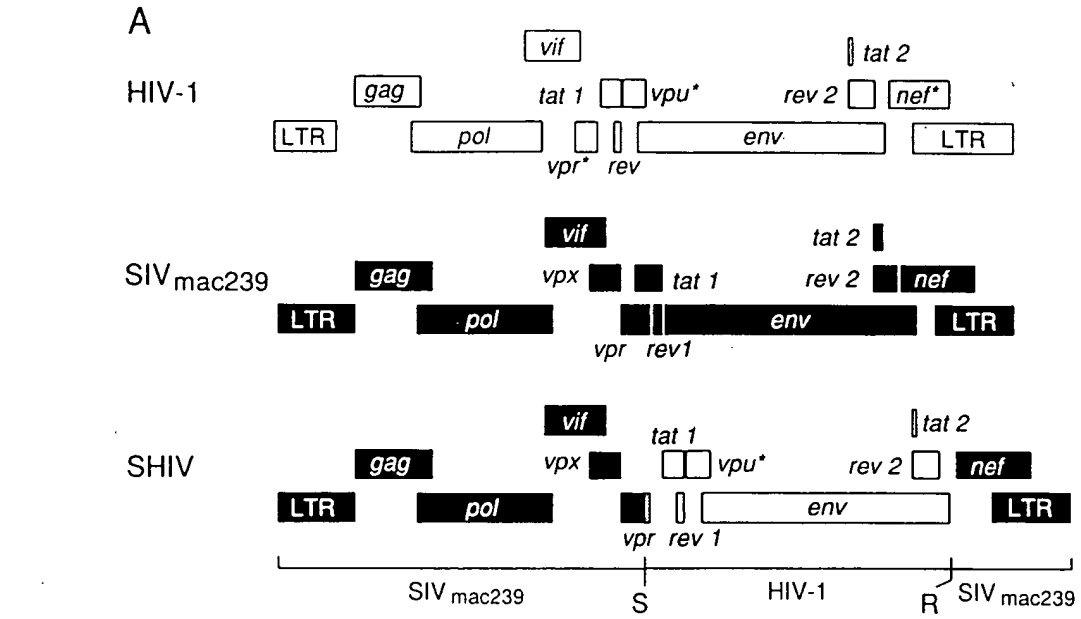
Typically, $2–4 \times 10^7$ peripheral blood mononuclear cells (PBMCs) were isolated from 15–30 ml whole blood from cynomolgus monkeys. Cells were isolated using Ficoll–Paque (Pharmacia) and resuspended in RPMI 1640 supplemented with 10% fetal calf serum and either phytohemagglutinin (PHA-C) (Boehringer-Mannheim) or concanavalin A (Con A, type IV, Sigma) at 5 μg/ml. Three to 5 days following PHA-C or Con A stimulation, the cells were washed and resuspended in RPMI 1640 with 10% fetal calf serum and 10 U/ml interleukin-2 (human recombinant, Boehringer-Mannheim). Two days later, PBMCs were infected with 1×10^5 reverse transcriptase units of virus derived from transfected CEMx174 cells. Three days after infection, PBMCs were washed and resuspended in fresh medium. Reverse transcriptase measurements in cell supernatants were made on days 4, 6, 9, and 13 following infection.

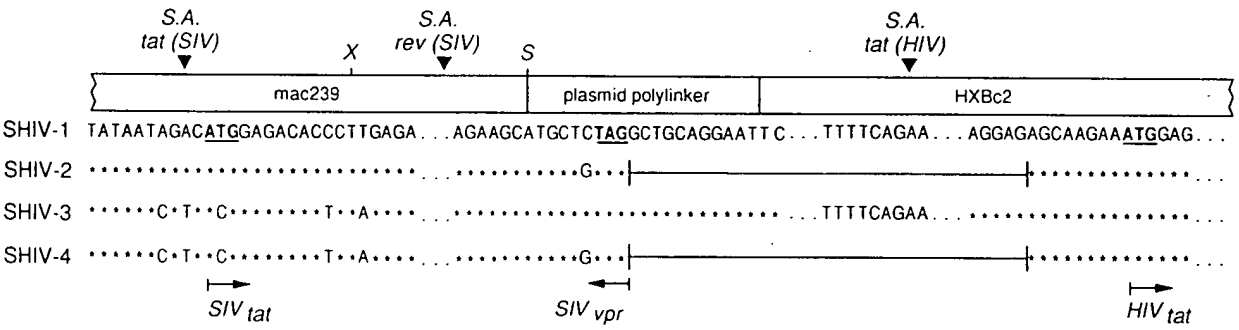
Preparation of Virus Stocks and $TCID_{50}$ Determination

Virus stocks for animal inoculation were prepared in cynomolgus monkey PBMCs and frozen as cell-free supernatants without additives at −70°C. The virus titer was determined by incubating 100 μl of thawed stocks, either undiluted or as 10-fold serial dilutions, in quadruplicate with 1×10^5 CEMx174 cells in 1 ml of medium. When cultures became confluent, cells were diluted 1/10. The wells were scored for the presence of syncytia after 2 weeks, and the 50% tissue culture infectious dose ($TCID_{50}$) in the virus stock calculated as described (38).

Immunoprecipitation of Infected Cultures

Approximately 2×10^6 CEMx174 cells were infected with HIV-1 (HXBc2 strain), $SIV_{mac}239$ (*nef* open), or chimeric viruses. The cultures were labeled overnight with [^{35}S]cysteine 1–2 days prior to the peak of syncytium formation, and cell lysates were precipitated either with serum from a HIV-1-infected AIDS patient or from a SIV_{mac}-infected rhesus macaque as described (30).





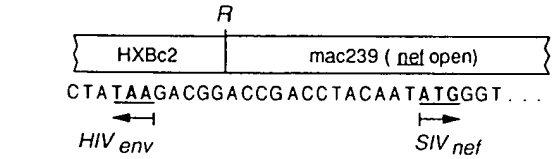


FIG. 1. Structure of the chimeric viruses. **A:** The genetic organization of the HIV-1 (HXBc2), SIV$_{mac}$239 (*nef* open), or SHIV chimeric viruses is shown, with HIV-1- or SIV$_{mac}$-specific sequences designated as white or black boxes, respectively. Genes that are defective in the strains utilized are denoted with an asterisk. The 5' SIV$_{mac}$/HIV-1 junction at the *Sph* I site (S) and the 3' HIV-1/SIV$_{mac}$ junction at the *Rsr II* site (R) are shown. The stippled 3' end of *vpr* of the SHIV virus represents sequences derived from the HIV-1 portion of the chimera that reconstitute the SIV$_{mac}$239 *vpr*. **B:** The details of the 5' SIV$_{mac}$/HIV-1 junction near the *Sph* I site (S) are shown for each of the SHIV chimeric viruses. The positions of the splice acceptors (S.A.) for the SIV$_{mac}$ *tat* and *rev* messages and for the HIV-1 *tat* message are shown above the figure, with the SIV$_{mac}$ *tat* initiation codon, SIV$_{mac}$ *vpr* stop codon and HIV-1 *tat* initation codon underlined and labeled with arrows below. The asterisks denote sequence identity and the dots represent sequences not shown. The horizontal bars represent sequence deletions. The X marks the position of an *Xba* I site in the SHIV-3 and SHIV-4 sequences. **C:** The details of the 3' HIV-1/SIV$_{mac}$239 (*nef* open) junction near the *Rsr* II site (R) are shown. The stop codon for the HIV-1 *env* and the initiation codon for the SIV$_{mac}$ *nef* are underlined and labeled with arrows.

Inoculation of Cynomolgus Monkeys with Chimeric Virus

Two male and two female cynomolgus monkeys (*M. fascicularis*) were inoculated intravenously with 1 ml of virus stock containing 7×10^3 $TCID_{50}$ of the SHIV-4 chimeric virus.

Virus Isolation from Inoculated Cynomolgus Monkeys

At 2 and 4 weeks following inoculation of cynomolgus monkeys, CD8-depleted, Con A-stimulated PBMCs were cultured from each animal and the level of SIV_{mac} gag p27 antigen in culture supernatants assessed as described (39). Culture supernatants positive for viral antigen were used to infect CEMx174 cells, which were labeled and used for immunoprecipitation as described above.

RESULTS

Chimeric Viruses

The goal of these studies was to create a replication-competent SIV/HIV-1 hybrid virus that expresses the HIV-1 envelope glycoproteins. The sequences used for the construction were derived from the pHXBc2 DNA, a clone prepared from the IIIB strain of HIV-1 (36), and the p239 SpSp 5′ and p239 SpE3′/nef-open plasmids derived from the $SIV_{mac}239$ strain of virus (12,35). The $SIV_{mac}239$ viral DNA was selected to be one of the parents for the recombinant virus as injection of cynomolgus or rhesus monkeys with either purified viral DNA or virus derived from this DNA resulted in both high levels of viremia and an AIDS-like disease (12,35, 40).

Construction of the appropriate chimeric molecules was complicated by significant differences in the regulatory genes of the two viruses as well as the complex genetic organization of the primate immunodeficiency viruses (5,41,42). Both HIV-1 and SIV_{mac} encode the regulatory genes *vif, vpr, tat, rev,* and *nef*. The regulatory gene *vpu* is specified only by HIV-1 (43–48), whereas *vpx* is found only in HIV-2 or SIV (49–51). The strategy used for construction of chimeric viruses was to replace the *tat, rev,* and *env* sequences of $SIV_{mac}239$ by the corresponding sequences of HXB2. The resultant viruses should contain the LTR *gag, pol, vif, vpx, vpr* and *nef* of SIV_{mac} and *tat, rev,* and *env* of HIV-1.

The original chimeric virus, designated SHIV-1 (SIV-HIV-chimeric virus-1), contains two *tat* splice acceptor sequences. The 5′ *tat* splice acceptor sequence is of SIV_{mac} origin whereas the 3′ *tat* acceptor sequence is derived from HIV-1 sequences. To minimize the possibility that the presence of two closely spaced splice acceptor sites might interfere with one another, derivatives of SHIV-1 were made that contain only the SIV_{mac} splice acceptor site (SHIV-2), only the HIV-1 splice acceptor site (SHIV-3) or neither splice acceptor site (SHIV-4) (Fig. 1). In the virus that lacks both *tat* splice acceptors, it is likely that the remaining SIV_{mac} *rev* acceptor substitutes for the *tat* acceptor.

Replication of Chimeric Viruses in Culture

The parental $SIV_{mac}239$ virus replicates well in the human $CD4^+$ B/T cell hybrid line CEMx174 (52). To determine whether chimeric SHIV DNAs produce infectious virus, CEMx174 cells were transfected with the parental $SIV_{mac}239$ as well as SHIV recombinant DNAs. Virus replication was monitored by measurement of the amount of the viral DNA polymerase (reverse transcriptase) released into the culture medium.

The data of Fig. 2 show that virus is produced from cultures that are transfected with all five DNAs. However, significant differences in the rate of appearance of reverse transcriptase in the medium were noted using different DNAs. Significant virus replication was evident by 9 days post-transfection in cultures treated with either the parental $SIV_{mac}239$ DNA or the SHIV-2 or SHIV-4 DNAs (Fig. 2A). Detectable levels of reverse transcriptase were not present in the cultures transfected with SHIV-1 or SHIV-3 DNAs until day 13 post-transfection (Fig. 2B). The relative delay in appearance of virus in the supernatant of cultures transfected with SHIV-1 or SHIV-3 DNAs as compared to those transfected with $SIV_{mac}239$, SHIV-2 or SHIV-4 DNAs was observed in several independent experiments. Despite this reproducible delay, the rates of replication of all four chimeric viruses were indistinguishable when similar amounts of virus harvested from the supernatant fluids of the transfected cultures were used to reinfect CEMx174 cells (data not shown).

The ability of SHIV-2 and SHIV-4 viruses to initiate infection in primary PBMCs derived from cynomolgus monkeys was examined. For these experiments the $SIV_{mac}239$, SHIV-2, and SHIV-4 viruses harvested from the supernatant fluids of transfected CEMx174 cells were incubated with PHA-1- or Con A-activated monkey PBMCs. Three days after infection with these viruses, the PBMCs were washed and resuspended in fresh medium. Virus replication

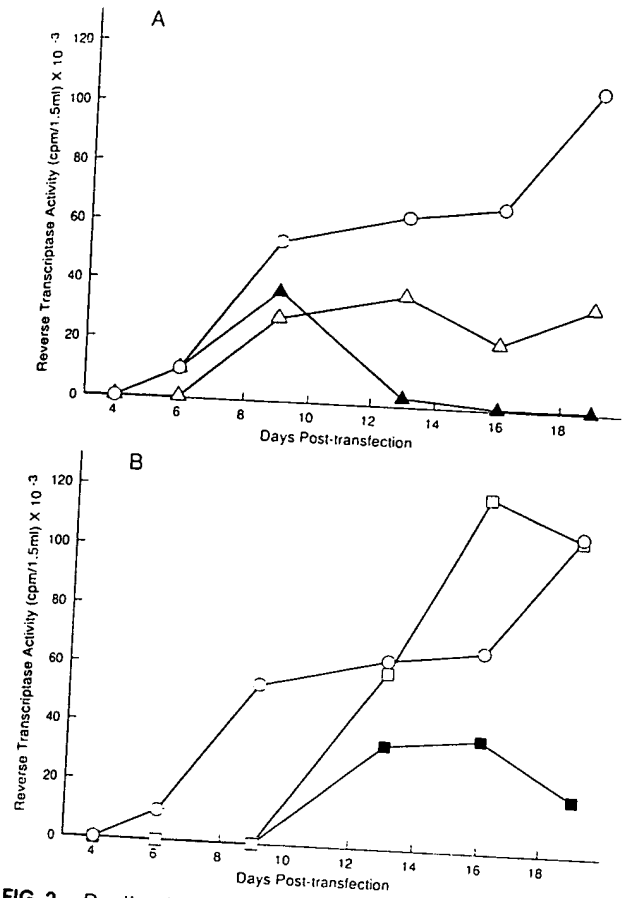


FIG. 2. Replication of chimeric viruses in CEMx174 lymphocytes. The reverse transcriptase activity in the supernatants of CEMx174 cells that had been transfected with proviral DNA of SIV$_{mac}$239 (*nef* open) (○) or chimeric viruses is shown. **A:** The CEMx174 cells were transfected with SHIV-2 (▲) or SHIV-4 (△) DNA. **B:** The CEMx174 cells were transfected with SHIV-1 (■) or SHIV-3 (□) DNA.

was measured by detection of reverse transcriptase activity in culture supernatant fluids.

The data of Table 1 show that all three viruses replicated well in cultures of PBMCs derived from cynomolgus monkeys. The rate of replication and amount of virus produced upon infection of the monkey PBMCs with either the SHIV-2 or SHIV-4 virus was similar to that obtained upon infection of the culture with SIV$_{mac}$239.

TABLE 1. *Reverse transcriptase activity (cpm/1.5 ml × 10^{-3}) in supernatants of cynomolgus monkey PBMCs*

Virus	Days after infection			
	4	6	9	13
SIV$_{max}$239 (*nef*-open)	33	45	30	61
SHIV-2	96	82	41	28
SHIV-4	57	76	17	34

Chimeric Nature of the Recombinant Viruses

The SHIV chimeras should produce gag and pol products of SIV$_{mac}$ and env proteins of HIV-1. The viral gag proteins of HIV-1 and SIV$_{mac}$239 can be distinguished by mobility differences on SDS–polyacrylamide gels, following precipitation with sera from HIV-1-infected humans or SIV$_{mac}$-infected monkeys. Such sera contain antibodies that cross-react with gag but not with env proteins (53). To determine whether the SHIV viruses that replicate in cynomolgus monkey PBMCs express both SIV$_{mac}$ and HIV-1 proteins, viruses harvested from the supernatant fluids of infected PBMC cultures were used to infect CEMx174 cells. As controls, CEMx174 cells were infected with SIV$_{mac}$239 (*nef* open) and HIV-1 (HXBc2) viruses. The infected cells were labeled with [^{35}S]cysteine, lysed, and the viral proteins precipitated with serum from a HIV-1-infected AIDS patient or serum from a SIV$_{mac}$-infected macaque. The precipitates were analyzed on SDS–polyacrylamide gels.

The data of Fig. 3A show that, as expected, both the human and monkey sera recognize gag proteins of the parental HIV-1 and SIV viruses. These proteins can be distinguished from one another by electrophoretic mobility of both the capsid proteins (HIV-1 p24 and SIV$_{mac}$ p27) and the gag precursor proteins (HIV-1 p55 and SIV$_{mac}$ p58). The HIV-1 serum recognizes the gp160 and gp120 env glycoproteins present in CEMx174 cells infected with HIV-1 but not the env proteins of cells infected with SIV$_{mac}$239. The anti-SIV$_{mac}$ serum recognizes the gp160 and gp130 env proteins present in cells infected with SIV$_{mac}$239 but not with the HIV-1 virus.

In these experiments the gag proteins present in cells infected with the SHIV-4 virus exhibited the electrophoretic mobility characteristic of SIV$_{mac}$ capsid proteins. The env proteins of these extracts were recognized by the anti-HIV-1 but not the anti-SIV$_{mac}$ serum. The electrophoretic mobilities of the env proteins present in cells infected with the SHIV-4 virus corresponded to those expected for the envelope glycoproteins of HIV-1. These experiments confirm that the SHIV-4 virus is chimeric and produces the gag proteins of SIV$_{mac}$ and the env proteins of HIV-1.

Infection of Cynomolgus Monkeys

The ability of one of the chimeric viruses, SHIV-4, to replicate in cynomolgus monkeys was exam-

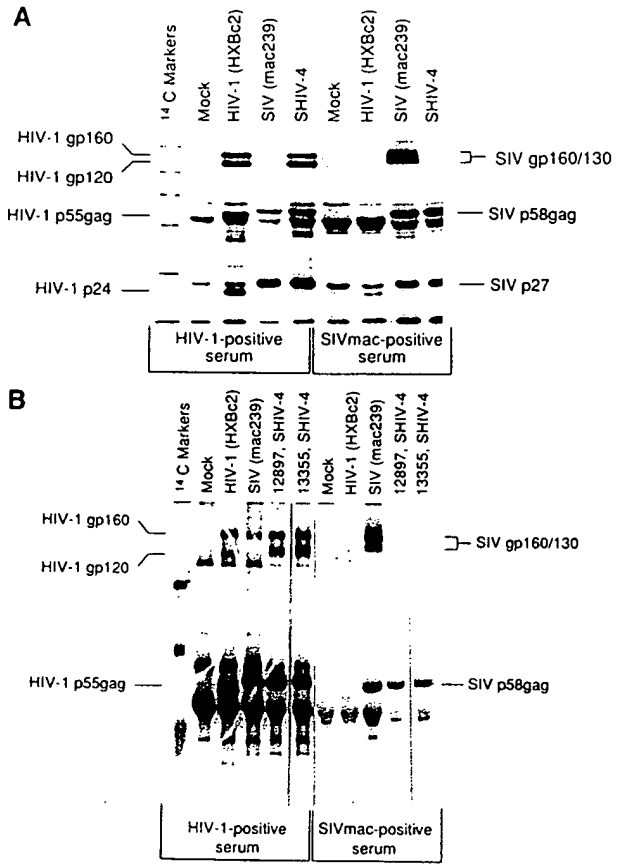


FIG. 3. Viral protein production of chimeric viruses. **A:** CEMx174 cells were infected with $SIV_{mac}239$ (*nef* open) virus or SHIV-4 virus that had been produced from cynomolgus monkey PBMCs. In parallel, CEMx174 cells were infected with HIV-1 (HXBc2 strain). Infected CEMx174 cells and uninfected (Mock) controls were labeled, lysed, and precipitated either with HIV-1-positive human serum or serum from a SIV_{mac}-infected macaque. The position of the HIV-1- and SIV_{mac}-specific *gag* and *env* products are marked. The molecular mass markers shown are 200, 96, 69, 46 and 30 kDa. **B:** CEMx174 cells were infected with the SHIV-4 virus isolated from cynomolgus macaques (numbers 12897 and 13355) at 2 weeks post-inoculation. In parallel, CEMx174 cells were infected with HIV-1 (HXBc2 strain) or $SIV_{mac}239$ (*nef* open) viruses, or mock-infected. The CEMx174 cells were labeled, lysed, and precipitated either with HIV-1-positive human serum or SIV_{mac}-infected macaque serum. The molecular mass markers shown are 200, 96, 69, and 46 kDa.

ined. For this experiment the SHIV-4 virus was grown in cynomolgus monkey PBMCs. The titer of virus produced in the PBMCs was determined using CEMx174 cells as targets. An amount of virus equivalent to 7×10^3 $TCID_{50}$ units was injected intravenously into four cynomolgus monkeys that were seronegative for SIV_{mac}. At 2 and 4 weeks post-infection PBMCs were isolated from the inoculated monkeys. The lymphocyte population was depleted for $CD8^+$ T cells and activated with Con-A as described previously (39). Virus was detectable by both p27 gag protein released into the culture fluid and by the formation of syncytia in activated PBMC cultures of all four monkeys at 2 and 4 weeks post-infection (data not shown).

The culture fluid obtained from the activated PBMCs of the four monkeys was used to infect CEMx174 cells. The cells were labeled with [^{35}S]cysteine and precipitated with anti-HIV-1 and anti-SIV_{mac} serum as described above. The viruses isolated from all four animals encoded gag precursor proteins that exhibited a mobility identical to that of the SIV_{mac} gag precursor protein, and encoded env proteins that were precipitated with HIV-1-positive but not SIV_{mac}-positive serum (Fig. 3B and data not shown).

DISCUSSION

These experiments demonstrate that a chimeric virus that expresses the gag and pol proteins of SIV_{mac} and the env proteins of HIV-1 can replicate efficiently in cynomolgus monkeys. The rate of appearance of virus in the peripheral blood mononuclear cells of infected monkeys is comparable to the rate of appearance of infection with the pathogenic strain of $SIV_{mac}239$.

The chimeric virus that replicates well in monkeys is predicted to express the vif, vpx, vpr, and nef regulatory proteins of SIV_{mac} and the tat and rev proteins of HIV-1. The *vpu* gene of HIV-1 present in the SHIV-4 chimera is defective as a consequence of a missense mutation in the initiation codon (43,47). These results demonstrate that restriction of replication of HIV-1 in cynomolgus monkeys is not due to determinants in *tat*, *rev*, or *env*.

This is the first report of high level replication of an HIV-1/SIV chimeric virus in monkeys. Several factors probably contribute to the ability of SHIV-4 to replicate efficiently in monkeys. The SHIV-4 virus contains functional SIV_{mac} *vpr* and *nef* open reading frames. Previous studies demonstrated that a functional *nef* of $SIV_{mac}239$ is required for high level replication and for disease induction in rhesus macaques (35). The *vpr* of HIV-2 and SIV_{mac} strains has been reported to facilitate replication of the virus in primary macrophage cultures (54–56). Since high virus loads appear to be one prerequisite for pathogenicity, the SHIV-4-infected monkeys will be monitored to assess the presence and time course of disease induction.

Shibata et al. (57) have also constructed a SIV$_{mac}$/HIV-1 chimeric virus that expresses the HIV-1 envelope glycoproteins. This virus is defective for both *vpr* and *nef*. The chimera described by Shibata et al. replicates in cultured monkey PBMCs. However, the rate of replication of this virus appears to be slower than that of the SIV$_{mac}$ parental virus (57). The absence of *vpr* and *nef* and suboptimal splicing patterns may account, at least in part, for this delay.

The presence of the HIV-1 *tat* splice acceptor near the 5′ SIV$_{mac}$/HIV-1 junction slowed the appearance of virus in cells transfected with the SHIV-1 or SHIV-3 DNA. It is likely that the presence of the HIV-1 *tat* splice acceptor results in inefficient expression of viral genes. The ability of virus harvested from CEMx174 cells transfected with proviruses containing the HIV-1 *tat* acceptor sequence to initiate efficient reinfection of CEMx174 cells probably indicates that selection for optimal splicing occurs rapidly in culture.

The experimental system described here (the infection of cynomolgus monkeys with the SHIV-4 virus) should provide a valuable model for study of the efficacy of anti-HIV-1 vaccines. The ability of such vaccines to induce protective immune responses in monkeys to infection by SHIV-4 should provide an indication of efficacy against viruses with HIV-1 envelope glycoproteins. The model can also be used to evaluate the ability of polyclonal and monoclonal antibodies to inhibit HIV-1 envelope function in animals. Therapeutics designed to inhibit the HIV-1 *tat*, *rev*, or *env* functions can also be evaluated in this model system.

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Exhibit 5

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Candidate Vaccines Protect Macaques against Primate Immunodeficiency Viruses

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ABSTRACT

The preclinical evaluation of the efficacy of potential vaccines against AIDS requires challenge models. The experimental infection of macaques with simian immunodeficiency virus, human immunodeficiency virus type 2 (HIV-2) or chimeric viruses have proven to be most valuable. The progress made using simian models to assess the efficacy and identify the correlates or mechanism of protection by whole inactivated virus, live attenuated virus or recombinant sub-unit vaccines is reviewed. It is possible to conclude from these studies that an effective AIDS vaccine is feasible. Furthermore, it is likely that these different vaccine approaches protect through distinct mechanisms. These results provide a scientific basis for the development and selection of suitable candidate human AIDS vaccines for testing in humans.

INTRODUCTION

MACAQUE MODELS have become an essential tool in the development of vaccines against acquired immunodeficiency syndrome (AIDS). A wide variety of models are now available in which prophylaxis and pathogenesis can be studied. The virology, immunology, and pathogenesis of simian immunodeficiency virus (SIV) infection of macaques is remarkably similar to that of human immunodeficiency virus (HIV-1) in humans. More importantly a number of significant observations originally made in macaques have subsequently been confirmed in HIV-infected patients. These close similarities between the viruses and disease of macaques and humans give confidence that vaccine strategies that are protective in macaques may have similar efficacy in human trials. There is now abundant evidence that vaccines based on viral subunits, inactivated whole virus, and live attenuated viruses are all capable of inducing significant protection in macaques. The strengths and potential weaknesses of these approaches and the mechanisms by which they operate is the subject of this brief article.

MACAQUE MODELS

Since the isolation of the first SIV from macaques in 1985,[1,2] a wide variety of models have been developed involving rhesus, cynomolgus, and pig-tailed macaques, sooty mangabeys, and African green monkeys. Strains of SIV designated SIVmac, SIVsm, SIVmne and SIVagm and strains of HIV-2 have been used in these species. The isolates of SIVmac, SIVsm, and SIVmne most frequently used for vaccine studies are derived from one species of African monkeys, sooty mangabeys. These viruses, like SIVagm, are nonpathogenic in their natural host, only causing AIDS-like disease when transmitted or adapted to various species of Asian macaques. The different combinations of viruses and animal species have led to a wide range of pathogenic consequences ranging from PBj14 variant of SIVsm, which causes an atypical and rapidly fatal enteric infection to SIVagm strains, which persistently infect African green monkeys without any apparent pathogenic outcome. In general, the most rapidly pathogenic combinations are those in which the viruses are detected at the highest titers in blood and plasma. However, there are exceptions. This range of infections closely mimics the pathogenic consequences of HIV-1 infections in humans.

A major limitation of macaques as models for HIV vaccine development was the inability of HIV-1 strains to infect simian cells, with the exception of certain strains of HIV-1 in *Macaca nemestrina*,[3] and the antigenic differences between SIV and HIV-1. However, the discovery by Shibata *et al.*[4] that genes of SIV could be replaced by those of HIV-1 and that the resulting SIV-HIV-1 chimeras could infect simian cells has partially overcome this problem. Furthermore, recent experiments,

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sometimes involving serial passage *in vivo*, have resulted in the development of SHIV strains that can induce profound CD4 depletion.[5–7] However, whether these viruses can induce a full range of AIDS pathogenesis is still uncertain. Nevertheless, such chimeric viruses will enable comparative evaluation of the efficacy of candidate HIV-1 envelope vaccines in a challenge model system.

A further strength of the macaque model was the demonstration that animals could be infected by a variety of routes: intravenous, intravaginal, intrarectal, and oral. An additional refinement of the macaque models for testing the efficacy of vaccines is that the timing and dose of the challenge can be accurately defined. This range of macaque models has been extensively exploited over the last 10 years. In order to provide a large amount of comparative data on the efficacy of various vaccine strategies, these studies have provided data not only on the potency, but also on the breadth of the protection induced. In interpreting the results of these studies, there has often been criticism of their inconsistency. However, more careful analysis of apparent inconsistencies often reveals that different results relate to different species, virus, route, or dose that has been used in testing the efficacy of a vaccine strategy. Complete protection against infection is clearly much easier to achieve in a model in which the virus replicates to only low levels. Nevertheless, even this level of protection is significant and could profoundly alter the course of the epidemic if translated into large scale human trials.

SUBUNIT VACCINES

Early attempts to protect macaques by vaccinating with recombinant envelope and Gag proteins were disappointing.[8–10] In these experiments, no attempt was made to measure virus load and detection of virus after challenge was assumed to indicate an ineffective vaccine regime. A notable exception was the protection of cynomolgus macaques against infection with SIVmne, by using recombinant vaccinia virus expressing gp160 followed by immunizations with recombinant protein.[11] Subsequent studies have revealed that many subunit vaccines incorporating envelope proteins without or without the addition of gag significantly reduce the virus load following challenge.[12–15] This effect is often more marked if the vaccination regime includes a priming step using live expression vectors such as vaccinia virus. In some of these studies, an inverse correlation between antibody titers and virus load has been demonstrated. However, this is not always so and a definitive experiment in which protection is passively transferred with antibody has not yet been performed. The protective immunity elicited by recombinant envelope vaccines alone seems limited and further refinements will be needed to increase their robustness. Several promising approaches, such as including multiple antigens, and using different priming and boosting antigens, and using more effective antigen presenting systems, are being actively pursued. In contrast, significant reductions in the virus load after vaccination with recombinant vaccinia virus expressing the Nef protein of SIV correlated with levels of precursor $CD8^+$ major histocompatibility complex (MHC) class I restricted cytotoxic T cells activity.[16] Targeted lymph node immunization using the envelope and core subunit antigens has protected rhesus macaques against SIV administered intrarectally,[17] but the protection is not reliable against vaginal challenge.[18] Recombinant envelope proteins delivered via alternative vectors, such as naked nucleic acid or adenovirus have also significantly decreased viral burden after challenge intravenously or intravaginally.[19,20]

A significant advance in recent years has been the ability to test the efficacy of candidate HIV-1 envelope vaccines in macaques using SHIV chimeras as challenge viruses. These studies have demonstrated that existing subunit vaccines based on T cell adapted strains of HIV-1 effectively protect macaques against infection with SHIV-4 carrying the homologus gp120 derived from the HXB2 clone of HIV-1LAI.[21,22] Protection against this chimeric virus has also been induced by vaccination with plasmid DNA followed by recombinant protein.[23] A different candidate HIV-1 envelope vaccine based on a low passaged European isolate, W61D, which has been tested in clinical trials may be more appropriate because this protein binds to CCKR5. The vaccine effectively protected 10 of 12 macaques from the homologus SHIV chimera bearing the W61D envelope, but was unable to protect a separate group of four cynomolgus macaques against challenge with $SHIV_{SF33}$ carrying the envelope of San Francisco isolate and sharing 82% amino acid identity with W61D gp120.[24,25] It is not yet clear whether the failure to protect against $SHIV_{SF33}$ was the result of antigenic divergence or its greater replicative capacity *in vivo*.

The mechanism by which these candidate HIV-1 envelope vaccines protect against SHIV chimeras has not yet been defined. There is some evidence that protection correlates with levels of neutralizing antibody, but passive transfer of these antibodies has not yet been attempted. Furthermore, in experiments where animals have been primed with replicating vectors, it is clear that substantial levels of cytotoxic T cell activity is present.[23]

These studies clearly demonstrate the capacity of subunit vaccines to induce protection in macaques against live virus challenge. In many cases the protection is limited to a significant reduction in virus load rather than complete elimination of detectable virus. There is also evidence that the protection may only be effective against antigenically closely related virus. Nevertheless, this degree of protection may be sufficient to delay the course of the AIDS epidemic and the ultimate significance of these encouraging results in macaque models can only be evaluated in phase 3 human efficacy trials.

INACTIVATED VIRUS VACCINES

The first vaccines to prevent SIV infection in macaques were formalin inactivated whole SIV administered with the appropriate adjuvant.[26,27] These exciting observations were soon repeated and extended in many other centers in the United States and Europe.[28–30] A wide variety of adjuvants and inactivants were used and vaccines were shown to be protective against a wide range of antigenically divergent viruses administered by mucosal and intravenous routes. Substantial protection was still detectable 24 weeks after vaccination. The striking success of these vaccines was instrumental in engendering optimism and encouraging manufacturers to develop vaccines against AIDS.

The potency of the protection was particularly impressive since no evidence of infection could be detected even by polymerase chain reaction (PCR).

An intensive search for the correlates of this potent immunity soon revealed that antiviral and neutralizing antibodies correlated with protection, but surprisingly the correlation with antibodies to host cell proteins present in the vaccine was significantly stronger.[31] Furthermore, macaques were protected by immunization with uninfected cells alone. It was subsequently shown that although the vaccines protected against challenge with SIV grown in human T cells (the substrate used to grow the vaccines) they did not protect against SIV grown in simian cells.[32,33] These unexpected observations gave rise to widespread disillusionment and exaggerated suggestions that the macaque models were inappropriate for studies of AIDS vaccines. As a result, the study of the efficacy of inactivated virus vaccines against AIDS was largely abandoned. Nevertheless, those who continued to study this unconventional mode of protection continued to make intriguing observations. Xenogeneic MHC class II, and to some extent class I, molecules were shown to protect against SIV grown in human T cells and this protection could be transferred with serum.[34,35] It was also shown that allogeneic simian peripheral blood mononuclear cells could protect against SIV grown in simian cells.[34] However, it has proved significantly more difficult to induce consistent protection with uninfected allogeneic cells than with xenogeneic cells.[36,37] Studies are currently underway to test SIV vaccines grown in transformed simian T cell lines against SIV grown in simian cells. The possibility that allogeneic or altered host cell proteins may induce protective responses and that these can be induced by virus grown in allogeneic cells still remains a viable option for AIDS vaccines in humans.

LIVE ATTENUATED VIRUS VACCINES

A further major impetus to AIDS vaccine development was provided by the discovery in 1992 that SIV with a deletion in the *nef* gene was attenuated and animals chronically infected with this virus were protected against challenge with wild-type SIV.[38] This observation has also been widely reproduced and extended in several other centers.[39–43] There is little doubt that the protection induced by attenuated SIV is significantly more potent than anything else yet observed in the field. Animals infected with attenuated virus are protected against high doses of virulent challenge virus administered intravenously, intrarectally or intravaginally. The immunity persists for at least 18 months and is effective against a wide range of antigenically distinct viruses, including aggressive pathogenic SHIV chimeras carrying unrelated env, tat and rev genes of HIV-1.[44] Furthermore, attenuated SIV protects against challenge with live virus infected cells.[40]

There are clearly major safety concerns about the use of live attenuated retrovirus vaccines. The attenuated virus will remain active within the vaccinee for their entire lifetime. Therefore ensuring that the attenuated phenotype is stable and that the integrated retrovirus proviral DNA will never activate oncogenes or inappropriate expression of normal genes will be a difficult and lengthy process. As with all attenuated viruses, some strains of SIV are overattenuated and insufficiently immunogenic. However, the pathogenic potential of SIV with attenuated genotype has already been demonstrated in neonatal macaques and a small proportion of normal adults.[45,46] Achieving the correct balance between immunogenicity and pathogenicity is difficult and so far there is no good pathogenesis model for HIV-1. In view of these concerns, the immediate contribution of the live attenuated viruses to AIDS vaccine development is the hope they offer that potent and persistent immunity is possible. While long-term studies of the safety of attenuated lentiviruses must continue a more immediate objective is to understand the mechanisms by which these viruses protect and to seek less hazardous means to reproduce their immunity. The macaque model is the only system in which these studies can be performed.

The mechanisms by which live attenuated SIV induces such potent protection remain enigmatic. The attenuated virus continues to replicate at low levels throughout the life of the individual. In a minority of rhesus macaques, although apparently not in cynomolgus macaques, the attenuated SIVC8 virus has repaired the deletion in the *nef* gene and coincidentally reverted to a virulent phenotype resulting in fatal AIDS-like disease.[47] However, if the revertant virus is reintroduced into a rhesus macaque infected with attenuated virus the animal remains resistant.[48] Thus, the immunity induced by live attenuated virus is unable to eliminate vaccine virus or to prevent the emergence and pathogenicity of revertant virus arising endogenously, but is fully effective against the same revertant virulent virus introduced exogenously. This observation is in striking contrast to experiences with other live attenuated virus vaccines, such as poliovirus, where revertant virus with virulent phenotype can arise in vaccinated children within 7 days, but is effectively controlled by the vaccine-induced immunity in most vaccinees.[49] Any explanation of the protective mechanisms induced by attenuated SIV must accommodate these unexpected findings.

Macaques infected with attenuated SIV develop a vigorous $CD8^+$ cytotoxic T cell response.[50] Neutralizing antibodies and T helper responses have also been detected. However, attempts to correlate these responses with protection have proved difficult partly because relatively few vaccinated, unprotected animals have been observed. Using more direct approaches that circumvent the weaknesses of *in vitro* assays has still not yielded unequivocal results. Passive transfer of antibody from animals that have been vaccinated with live attenuated SIV and that have resisted challenge with virulent virus, has failed to protect recipients.[51] In contrast, transfer of antibody from macaques infected with a macrophage tropic and apparently attenuated strain of SIV_{SM} conferred protection on a proportion of the recipients.[52] Transfer of immune T cells is not practical between outbred macaques and in these circumstances, specific depletion of T cell subsets is a classic approach to defining their role in immunity. However, substantial depletion of circulating $CD8^+$ or total lymphocytes using monoclonal antibodies did not abrogate the protection induced by attenuated SIV,[53] whereas similar levels of depletion significantly affected the ability of macaques to control early infection with a SHIV chimera.[54] These results do not support a major role for cellular immunity in the protection induced by live attenuated virus. Likewise, studies on the production of CD8 inhibitory factors and the *in vitro* susceptibility of peripheral blood mononuclear cells have not yet indicated that soluble inhibitors or retroviral interference are significant factors.

CONCLUSION

Macaque models have provided convincing evidence that vaccination against lentivirus infection is achievable. They have also revealed mechanisms by which inactivated virus and some subunit vaccines protect. The efficacy of currently available candidate HIV-1 envelope vaccines can be compared in macaques, using appropriate SHIV chimeras to test potency and breadth of protection. Ultimately, only phase 3 efficacy trials in humans will establish if the modest protection induced by subunit vaccines is sufficient to alter the course of the AIDS pandemic. If they are not, alternative strategies are available for development. We should use the abundant data emerging from animal models wisely to guide our choice of vaccine for phase 3 trials.

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Exhibit 6

Attorney Docket No. 04012.0188

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Prieels April___, 1998

Serial No.: 08/909,879 Group Art Unit No.: 1818

Filed: August 12, 1997 Examiner: L. Smith

For: VACCINE COMPOSITION CONTAINING ADJUVANTS

Assistant Commissioner of Patents

Washington, D.C. 20231

DECLARATION OF DR. GERALD VOSS

1. I, Dr. Gerald Voss, a citizen of Germany and residing at , 12 Rue de la Barre, Grez-doiceau, Belgium, state and declare the following with respect to the invention described and claimed in U.S. Patent application No. 08/909,879 (Attorney Docket No. 04012.0188), entitled "VACCINE COMPOSITION CONTAINING ADJUVANTS."

2. I have received the following academic qualifications:

- Degree in Biology '83-'85, University of Freiburg, Germany.
- Master's degree in Biology (German diploma) '89, University of Goettingen, Germany
- Ph.D. in Biology '92, University of Goettingen and German Primate Centre, Goettingen.

I undertook a postdoctoral research position in Medicine, '93-'96, Harvard Medical School, Boston, USA. I joined SmithKline Beecham Biologicals in 1996, as a research group leader, specifically as an immunologist in the HIV, malaria, Dengue and adjuvant evaluation programs.

3. I have read and am familiar with the Office Action dated November 24, 1997, issued in the above captioned action.

4. I understand that the Examiner rejected the claims directed to a vaccine composition containing HIV and FIV antigens because, according to the Examiner, the specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims. In my opinion, and as explained in the response, the specification enables a person skilled in the art to make the HIV and FIV vaccine compositions of the subject invention and to use them as effective vaccines against HIV and FIV infections. Several studies and tests conducted by SmithKline Beecham Biologicals or under the Company's direction, which are described in the paragraphs below, support this conclusion.

The role of HIV-specific CTL in HIV infection episodes

5. In recent years much experimental work has been performed with the aim of identifying particular immunological responses which are important in the immunological control of HIV infections. In my opinion recent published work has suggested the important role of CTL in the control of HIV infections.

6. For example, CTL's have been shown to be activated *in vivo* to high levels, which activation coincides with a reduction of viraemia (1, 2, 3). Further suggestive evidence about the important role of CTL include observations that HIV-specific CTL are crucial in the maintenance of the asymptomatic phase of infection, before the development of the acquired immune deficiency syndrome (AIDS). Thus long-term non-progressors have high levels of HIV specific CTL, whereas HIV specific CTL are lost during progression to AIDS (4, 5, 6, 7, 8, and 9).

7. CTL have been associated with virus clearance from infected individuals and also with clinical protection from a number of viral infections. Accordingly, a number of studies have suggested that vaccine-induced protection against simian and feline immunodeficiency viruses, is correlated with the induction of strong antigen-specific CTL responses (10, 11).

8. Thus, the induction of anti-HIV CTL is not merely the induction of an immune response. It is the induction of the type of immune response which is postulated to be a major correlate of HIV prophylaxis or therapy.

The choice of the experimental animal model for the investigation of putative HIV vaccines

9. During recent years a number of authors have attempted to identify the most relevant model for testing of potential prophylactic and therapeutic anti-HIV vaccines. For example, the chimpanzee has been used in a number of early HIV studies because of its susceptibility of HIV infections (17, 18). However, the observation that chimpanzees do not develop AIDS-like symptoms has led to suspicion that these studies may not be the most relevant model for investigating the immuno-prophylactic and therapeutic role of anti-HIV vaccines. The chimpanzee model has also been criticised for several other reasons including, ethical and regulatory difficulties; high costs and limited supply of chimpanzees; and also high costs of safe and sufficiently large containment facilities.

10. Currently, the best model to study anti-HIV vaccines is the simian-human immunodeficiency virus (SHIV) infection of the rhesus monkey. The chimeric SHIV expressing the HIV-1 Env protein allows for the evaluation of HIV-1 vaccines in a simple and relevant animal model (12). Infected animals mount a vigorous immune response similar to those observed in infected humans, including CD8+ CTL (13). In contrast to the HIV-1 infection of chimpanzees, infection of rhesus monkeys with some SHIV strains induces typical AIDS-like symptoms (14). The most prominent feature of this disease induction is a rapid CD4+ cell decline (15, 20). Molecular clones of the pathogenic virus have now been characterised (16).

11. The SHIV/rhesus monkey model of HIV infection provides the most feasible model at the present time for the testing of potential prophylactic and therapeutic HIV vaccines. Furthermore, the SHIV rhesus monkey model does not share the other

criticisms as those experienced with the chimpanzee model to the same extent. Rhesus monkeys are smaller and easier to handle, are more extensively available at reasonable cost.

Experimental evidence performed with vaccines of the present invention

12. A number of experiments have been carried out which investigate the combination of 3D-MPL and QS21 for use as vaccine adjuvants for the development of a human anti-HIV vaccine. Such experiments are described herein and include vaccination studies in murine and rhesus monkey models, and also phase I human trials.

The generation of HIV-specific CTL in the murine model and in human vaccine trials.

13. The generation of HIV-specific CD8+ CTL is thought to be crucial for any successful anti-HIV vaccine (see above). The following experiments describe the induction of potent anti-HIV CTL responses in the murine model. Thus, vaccine adjuvants of the present invention, comprising 3D-MPL and QS21, are capable of stimulating one type of immune responses which have been postulated to be crucial for any successful anti-HIV vaccine.

14. Groups of 8 mice were vaccinated on two occasions with the vaccine formulations as described in table 1. The results shown in table 2, and figures 1, and 2, demonstrate that strong anti-HIV humoral and CTL responses were induced with the 3D-MPL/QS21 adjuvants. These results are summarised in table 3.

15. A phase I study, to investigate human application of an HIV vaccine, has been performed. Such trials are primarily designed for the purpose of measuring the safety, reactogenicity, and volunteer tolerance of the vaccine candidate. The phase I trial was successful in that it demonstrated the immunogenicity and safety of such an HIV vaccine. Work is now continuing in this area with the aim of future clinical studies.

16. During the phase I clinical study, the opportunity arose to make initial, preliminary, attempts to detect HIV-specific CTL in the human vaccinated volunteers. The detection of CTL, which was not the aim of the study, was unsuccessful, but was attempted because of the unique opportunity of having source of post HIV-vaccination human PBMC.

17. The observation that no HIV-specific CTL were detected in the PBMC at a time point 4 weeks after vaccination was not surprising. Reasons as to why circulating HIV-specific CTL were not detected include the fact that any CTL that were induced could have selectively populated a particular region of the body (*e.g.* lymph nodes) which precluded their detection in circulating PBMC. The timing of the sample schedule was not ideal for the detection of circulating CTL, as CTL may have been present at times other than this single time point 4 weeks after vaccination. Moreover, this particular attempt to detect CTL only involved a very limited number of individuals (4-5 individuals out of a group of 10 volunteers), such that the possibility of the induction of CTL in individuals vaccinated in a larger study could not be precluded. Furthermore, it was recognised that the CTL detection assay was sub-optimal due to the lack of positive control samples. Despite all of the above, as the PBMC were available at this time it was thought to be worthwhile to try to detect HIV-specific CTL.

18. Following the successful completion of the phase I trial, and the observation that the vaccine was immunogenic and well tolerated, SmithKline Beecham continues to work and invest in this area, including future clinical trials.

Experimental vaccination and challenge studies using the SHIV Rhesus monkey model.

19. In the experimental studies performed, vaccination regimes comprising 3D-MPL and QS21, and the well known and characterised $gp120_{W6.1D}$ (recombinantly produced in CHO cells using a sequence derived from the HIV strain W6.1D (19)) were successful in inducing strong *in vitro* virus neutralising humoral responses. The

vaccination regime induced protection from challenge with the homologous $SHIV_{WR1D}$ virus, in two of the four vaccinees. This homologous virus strain initiates a clinical infection which does not progress to AIDS-like symptoms, it is therefore a valid model for investigating vaccine prophylaxis of HIV infection. All of the control animals became infected (n=4). The formulations used and the results obtained are summarised in tables 4, 5 and 6.

20. Assays designed to detect HIV-specific CTL responses did not generate any interpretable data, this was because of the lack of a fully characterised assay system with appropriate control samples.

The vaccines of the present invention stimulate strong Th1-type cytokine production

21. Experiments have been performed to investigate the effect of QS21/3D-MPL on the stimulation of IFN-γ (Th1-type cytokine), and the data is presented in the patent specification (page 9, 1.4 results). This data shows that the combination of the two adjuvants is capable of acting in a synergistic fashion in the induction of high levels of IFN-production

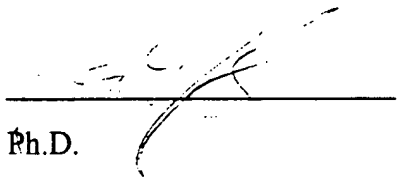
22. CTL responses are normally associated with strong Th1-type responses, thus CTL's can be considered to be a subset of TH1-type responses. As discussed above, HIV-specific CTL are thought to be correlates for protection, it may also be that other Th1-type effector components play a role in protection in combination with the CTL. As QS21 and 3D-MPL containing vaccines are capable of inducing both CTL, and other Th1-type responses (as disclosed in the patent application specification), it is expected that the crucial mixture CTL/Th1 effector components will be generated by vaccination with HIV vaccines containing QS21 and 3D-MPL.

Conclusions

23. In conclusion, vaccines of the present invention, namely combinations of 3D-MPL and QS21, together with HIV antigen, have been shown to have efficacy in one

of the best animal models currently available for the investigation of potential prophylactic HIV vaccines. Vaccines of the present invention, thus, provide a viable and potentially successful anti-HIV vaccine suitable for human use.

24. I declare that all statements made herein based on my own knowledge are true and that all statements based on information and belief are believed to be true; and further that the statements are made with the knowledge that wilful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such wilful false statements may jeopardize the validity of the above application or any patent issued therefrom.



Date: April ___, 1998 Gerald Voss, Ph.D.

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20. Reimann, K.A. *et al.* (1996) An env gene derived from a primary human immunodeficiency virus type 1 isolate confers high *in vivo* replicative capacity to a chimeric simian/human immunodeficiency virus in rhesus monkeys. J Virol., 70, 5, 3198-3206.

Mouse model data

Table 1, *Vaccine formulations*

Each group consisting of 8 mice vaccinated twice and samples taken 7 days post second vaccination (V2).

Group	*Antigen*	*Adjuvant formulation*
1	-	SB62, 3D-MPL (5μg), QS21 (5μg)
2	gp120 (5μg)	3D-MPL (5μg), QS21 (5μg)
3	gp120 (5μg)	Alum (50μg)

NB, SB62 is an oil in water emulsion comprising squalene, α-tocopherol, and TWEEN80™

Table 2, *Serological results*

Group	*ELISA titres (GMT)*	*IgG1 %*	*IgG2a %*	*IgG2b %*
1	0	0	0	0
2	45910	22	48	30
3	874	100	0	0

Table 3, *Summary table of results in the murine model*

Group	*Humoral Immunity*		*Cellular Immunity*					
			Lymph nodes			*Spleen*		
	ELISA	*IgG2a*	*Prolif*	*IL-2*	*CTL*	*Prolif*	*IL-2*	*CTL*
1	0	ND	0	0	0	0	0	0
2	++	++	++	+	++	+++	+	++
3	0	0	++	+/-	0	+	+	0

Figure 1. CD8+ CTL detected in Popliteal lymph node cells [illegible]

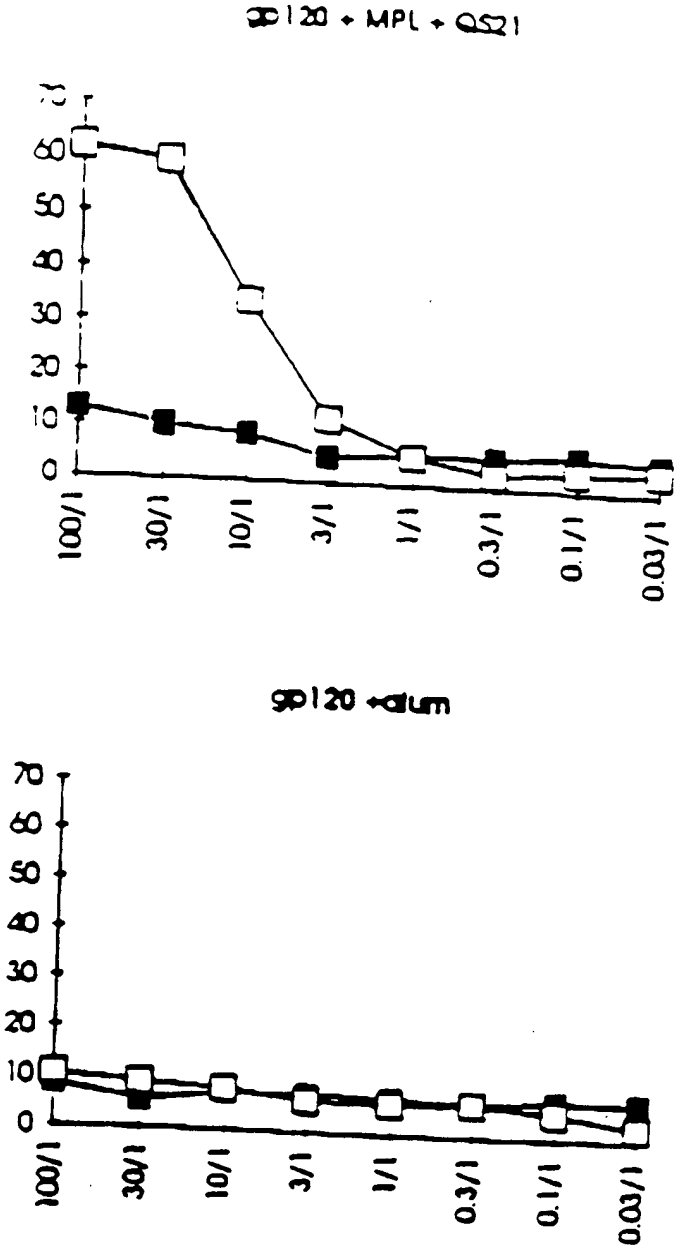
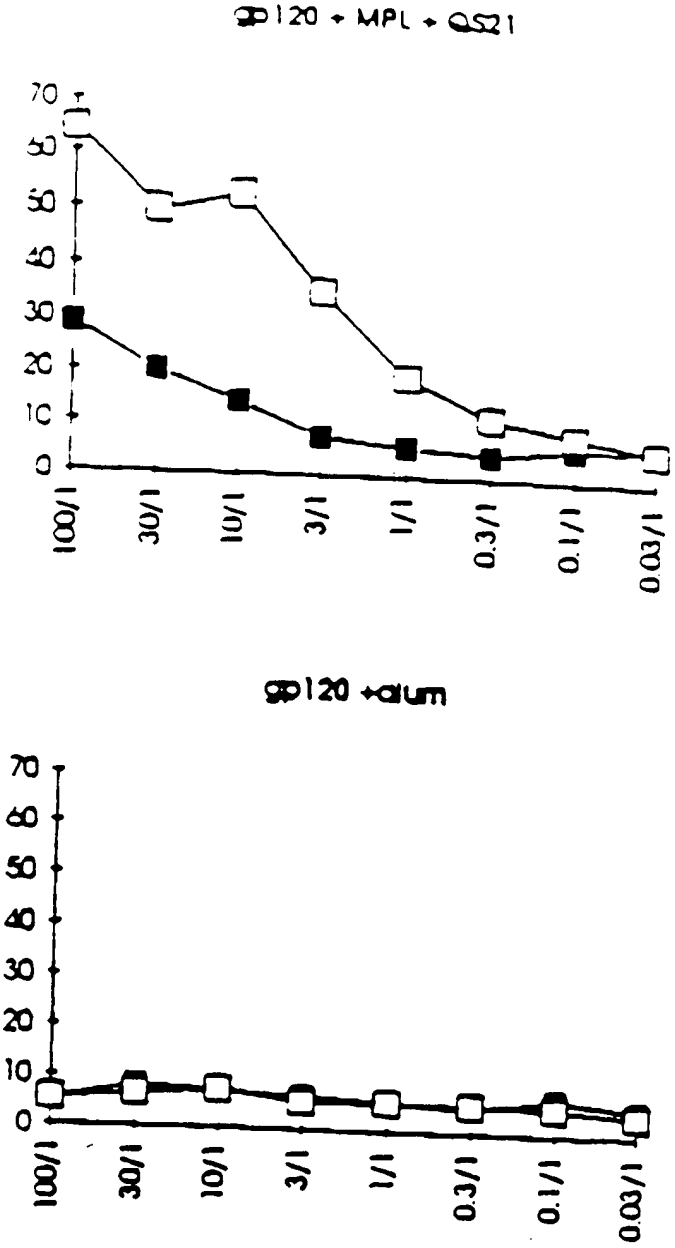


Figure 3 CTL - CTL detected in spleen cells 7 days post VV



Rhesus monkey data

Rhesus monkeys were vaccinated at 0,1,3,12,19 months and challenged (28 days post V5) with homologous virus strain $SHIV_{W6.1D}$.

Table 4, *Vaccine formulations*

Group	***Antigen***	***Adjuvant formulation***
A	gp120 (100μg)	QS21 (50μg), 3D-MPL (50μg).
Control	-	-

NB. Vaccinations number 4 and 5 also contained small unilamenar liposomes. In house data shows that the addition of the liposomes does not significantly affect the immunological responses observed with after vaccination compared to 3D-MPL/QS21 alone. The function of this addition is to reduce reactogenicity and to prolong stability of QS21 during long-term storage.

Table 5, *Serology results post V2 and V3.*

The results presented constitute anti-gp120 titres as measured by ELISA and reciprocal dilutions of the highest dilution of serum to neutralise virus growth in vitro (VN).

Group	***Animal***	***2 weeks post V2***		***2 weeks post V3***	
		anti-gp120	*VN*	*anti-gp120*	*VN*
A	*9150*	59310	400	45400	1600
	9175	10227	100	14216	400
	9208	16393	200	21900	400
	9214	5788	100	9595	200
	mean (+/- sem)	22930 (+/- 12320)	200 (+/-70)	22778 (+/- 7957)	650 (+/-320)

Table 6. *Summary table of Rhesus monkey serology at time of viral challenge and outcome of challenge.*

Group	***Animal***	***Serology***		***Outcome to homologous challenge***
		anti-gp120	VN	
A	9150	25600	320	Protected
	9175	51200	640	Protected
	9208	25600	640	Infected
	9218	25600	320	Infected
Control	AA002	<20	40	Infected
	J040	<20	40	Infected
	L146	<20	<40	Infected
	Y005	<20	40	Infected

Exhibit 7

Attorney Docket No. 04012.0188

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Prieels _____, 1999

Serial No.: 08/909,879 Group Art Unit No.: 1648

Filed: August 12, 1997 Examiner: L. Smith

For: VACCINE COMPOSITION CONTAINING ADJUVANTS

Assistant Commissioner of Patents

Washington, D.C. 20231

DECLARATION OF DR. GERALD VOSS

1. I, Dr. Gerald Voss, a citizen of Germany and residing at , 12 Rue de la Barre, Grez-doiceau, Belgium, state and declare the following with respect to the invention described and claimed in U.S. Patent application No. 08/909,879 (Attorney Docket No. 04012.0188), entitled "VACCINE COMPOSITION CONTAINING ADJUVANTS."

2. I have received the following academic qualifications:

- Degree in Biology '83-'85, University of Freiburg, Germany.
- Master's degree in Biology (German diploma) '89, University of Goettingen, Germany
- Ph.D. in Biology '92, University of Goettingen and German Primate Centre, Goettingen.

I undertook a postdoctoral research position in Medicine, '93-'96, Harvard Medical School, Boston, USA. I joined SmithKline Beecham Biologicals in 1996, as a research group leader, specifically as an immunologist in the HIV, malaria, Dengue and adjuvant evaluation programs.

3. I filed a declaration in the subject application on May 22, 1998, in relation to my experience with HIV vaccines of the present invention, and showing therein the protection of Rhesus monkeys from experimental HIV infection by administration of vaccines of the present invention.

4. I have read and am familiar with the Office Action dated August 12, 1998, issued in the above captioned action. I understand that the Examiner has again rejected the claims, the objection being based at least in part on the examiners assertion that HIV specific CTL induced by the vaccine was not shown to reduce viral load or viral burden, or slow progression to AIDS.

5. In my opinion the subject patent and additional data described in my declarations clearly show that administration of the vaccines of the present invention are capable of (a) inducing protection from infection, or (b) reducing viral burden, and delaying detection of infection in those animals that are not fully protected.

6. I would like to note that the measurement of viral burden or viral load, is an assay which is commonly performed in investigative studies to measure any ameliorative effects of a therapeutic strategy which fails to provide protection. Accordingly, in animals which are protected from infection, there is no detectable viral burden or viral load. Likewise, in animal models which exhibit progression to AIDS-like symptoms, vaccinees that are protected, and therefore, do not show any viral load or viral burden, do not progress to AIDS in the absence of infection.

7. In the Rhesus monkey SHIV model described in my previous declaration, the vaccines comprising QS21 and 3D-MPL adjuvants have been shown to induce protection in two out of four vaccinees. Thus, in the two monkeys that were protected there was no detectable viral burden or viral load. This protection from infection was observed despite the lack of interpretable CTL data.

8. Also, the monkeys that were infected (2 out of 4) did in fact show a reduced viral burden as evidenced by delayed HIV Polymerase Chain Reaction (PCR) and reduced virus isolation (Quantative Virus Isolation (QVI)) in comparison to negative control animals. These results were published recently in Mooij *et al.*, 1998, AIDS, 12:F15-F22. A copy of this article is provided in Annex I.

9. In this paper, the group comprising gp120, 3D-MPL, and QS21 is group A. The results are summarised in Figure 1 (Cytokine assays, including IFNγ), Table 2 (humoral responses) and Table 3 (virus status post-challenge).

10. Also described in the same paper is a group wherein the adjuvant comprised QS21 and 3D-MPL in the form of an oil in water emulsion (Group B). This vaccine formulation resulted in the protection of all four animals of Group B from infection without any detectable viral load or viral burden (Table 3).

11. Furthermore, a third group of vaccinated animals (group C) is described in this article which were vaccinated with gp120 antigen together with QS21 and 3D-MPL in the form of an oil in water emulsion, this vaccine being given after a previous vaccination experiment with an unrelated weak adjuvant. This experiment demonstrated the ability of the adjuvant of the present invention to improve a previously existing weak anti-HIV immune response. All four of these animals were protected from viral challenge after the third administration of the QS21 and 3D-MPL containing vaccine.

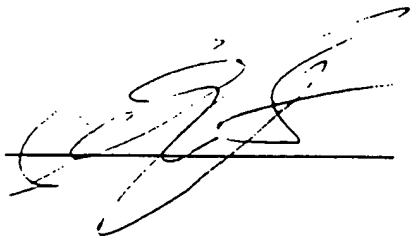
Conclusions

12. In conclusion, vaccines of the present invention, namely combinations of 3D-MPL and QS21, together with HIV antigen, have been shown to have efficacy in one of the best animal models currently available for the investigation of potential prophylactic HIV vaccines.

13. The Mooij *et al.* reference, therefore, describes twelve vaccinated animals which received a vaccine comprising HIV antigen, QS21 and 3D-MPL, 10 of which were protected from infection in the SHIV rhesus model. The remaining two animals showed reduced viral load and delayed onset of infection.

14. I declare that all statements made herein based on myown knowledge are

true and that all statements based on information and belief are believed to be true; and further that the statements are made with the knowledge that wilful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such wilful false statements may jeopardize the validity of the above application or any patent issued therefrom.



Date: January 15, 1999 Gerald Voss, Ph.D.

Exhibit 8

A clinically relevant HIV-1 subunit vaccine protects rhesus macaques from *in vivo* passaged simian–human immunodeficiency virus infection

Petra Mooij, Mike van der Kolk, Willy M.J.M. Bogers, Peter J.F. ten Haaft, Peter Van Der Meide, Neil Almond*, Jim Stott*, Marguerite Deschamps†, Dominique Labbe†, Patricia Momin†, Gerald Voss†, Paul Von Hoegen†, Claudine Bruck† and Jonathan L. Heeney

Objectives: To investigate whether immunization with recombinant HIV-1 envelope protein derived from a clinical isolate could protect macaques from infection with an *in vivo* passaged chimeric simian–human immunodeficiency virus (SHIV).

Design and methods: A total of 16 animals were studied from which three groups of four animals were immunized with vaccine formulations of the CC-chemokine receptor-5-binding recombinant gp120 of HIV-$1_{W6.1D}$. Four weeks after the last immunization, all 16 animals were intravenously challenged with *in vivo* passaged SHIV derived from the same HIV-1 group B clinical isolate (W6.1D) as the vaccines.

Results: Vaccine protection from infection was demonstrated in 10 out of 12 macaques immunized with recombinant gp120. Complete protection from infection was achieved with all of the animals that received the SBAS2–W6.1D formulation, a potent inducer of both T-cell and humoral immune responses. Partial protection was achieved with SBAS1–W6.1D, a formulation based on immunomodulators known to induce T-cell responses in humans. In vaccinated animals that were infected, virus load was reduced and infection was delayed.

Conclusions: In a relatively large number of primates, vaccine efficacy was demonstrated with a clinically relevant HIV-1 vaccine. These results reveal that it is possible to induce sterilizing immunity sufficient to protect from infection with SHIV which was passaged multiple times *in vivo*. Our findings have implications for current HIV-1 clinical vaccine trials and ongoing efforts to develop safe prophylactic AIDS vaccines.

AIDS 1998, 12:F15–F22

Keywords: Chimeric simian–human immunodeficiency virus, HIV-1 recombinant gp120, vaccine, rhesus macaque, protection

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Introduction

HIV, the cause of AIDS, is estimated to have resulted in more than 3 million new infections world-wide last year [1]. The great majority of these new infections are attributable to HIV-1, and currently there is no effective vaccine. The biological complexities of developing an effective HIV-1 vaccine require preclinical evaluation in non-human primates [2,3]. Until recently, the only non-human primate model for HIV-1 vaccine efficacy testing was the chimpanzee [4].

Subunit HIV-1 vaccines based on laboratory strains of HIV-1 have demonstrated efficacy in chimpanzees challenged with HIV-1 [5–7], but study groups were most often groups of two animals [4]. Although chimpanzee studies have had and will continue to have a profound importance as studies of 'proof of principle', there are still a number of important unresolved issues concerning the use of subunit vaccines, in particular the problem of relatively poor immunogenicity of the HIV-1 envelope. For instance, it is generally believed that effective HIV-1 vaccines must be capable of inducing strong cell-mediated immune responses as well as neutralizing antibodies in large outbred populations [3]. Such immunogenicity evaluation combined with efficacy requires comparative testing of various preparations in a model system that permits the practical use of larger groups of animals.

Inclusion of HIV-1 envelope antigen(s) in candidate vaccine strategies is thought to be a necessary component of a prophylactic HIV-1 vaccine [2,4]. For the purpose of addressing immunological vaccine issues related specifically to envelope, chimeric simian–human immunodeficiency viruses (SHIV) have been developed that express the envelope of HIV-1 and yet are infectious for various macaque species such as the rhesus macaque (*Macaca mulatta*) [8,9]. Although the majority of SHIV are non-pathogenic, their use provides new opportunities to determine the efficacy of vaccines for protection from infection rather than protection from disease.

First to study immunogenicity, and second to evaluate efficacy, we undertook a two phased study in outbred *M. mulatta*. In the first phase, a CC-chemokine receptor (CCR)-5-binding subunit gp120 of a clinical clade B HIV-1 isolate was used to compare the immune responses induced with two different adjuvant formulations. Each of the two different adjuvants were designed to induce proportionally different humoral/cellular immune responses. In the second phase, when the immune responses elicited by these two different adjuvant formulations were known, a third group was added, and all animals vaccinated plus additional naive control animals were challenged with an *in vivo* titrated stock of SHIV that had been derived by multiple serial passage in rhesus monkeys.

Materials and methods

Immunization

In the first phase of the study, two groups of four animals each were immunized intramuscularly in the upper leg on weeks 0, 4 and 12 with recombinant $gp120_{W6.1D}$ antigen derived from HIV-1 clone 32H isolated from a Dutch AIDS patient [10]. This antigen was formulated in either adjuvant SBAS1, consisting of saponin QS21 (*Quillaja saponaria* 21) and monophosphoryl lipid A (MPL-A; group A), or SBAS2, consisting of saponin QS21, MPL-A and emulsion (group B) [11]. Each dose consisted of 100 µg in a volume of 0.5 ml. In the second phase of the study, an extra group of four animals was added (group C). This third group had previously been immunized in another study with the same gp120 antigen at the same intervals (0, 4 and 12 weeks), but with an inferior unrelated adjuvant that induced very weak immune responses (data not shown). Group C was added to determine whether the SBAS2 adjuvant formulation was capable of improving previously weak immune responses, and if so, to see whether protective immunity could be achieved. All animals in groups A, B, and C received two additional boosts on weeks 47 or 51 and 75: group A with SBAS1–W6.1D and groups B and C with SBAS2–W6.1D. Four non-immunized naive animals served as controls (group D). Captive-bred mature (4–5 years old) outbred rhesus macaques were housed at the Biomedical Primate Research Centre (Rijswijk, The Netherlands).

Cell-mediated immune responses

Two weeks after the second and third immunizations, 8 weeks after the third, and 2 weeks prior to challenge, freshly isolated peripheral blood mononuclear cells (PBMC) from each monkey were assayed for $gp120_{W6.1D}$-specific [ARP-648; Medical Research Council (MRC) AIDS Reagent Programme, Potters Bar, Hertfordshire, UK] T-cell responses. Enumeration of antigen-specific cytokine-secreting [interferon (IFN)-γ, interleukin (IL)-2, and IL-4] cells by ELIspot assay as well as antigen-specific lymphocyte proliferation assays were performed as previously reported [12]. In brief, 1 µg recombinant gp120 of the clinical isolate W6.1D (MRC) was used as antigen-specific stimulator; medium alone was used as negative background control. Concanavalin A (5 µg/ml) was used as mitogen stimulator (positive control) to test responsiveness of the cells. Cytotoxic T-cell (CTL) responses were assayed as described previously [13] using 47 overlapping 20-mer peptides homologous for the entire HIV-1 $gp120_{W6.1D}$. Peptide pools used to analyse specific cytotoxicity were grouped as follows: pool A contained peptides 1–16, pool B contained peptides 17–31, and pool C contained peptides 32–47 of HIV-1 $gp120_{W6.1D}$. Assays were based on the use of at least three different effector : target ratios. Responses were

... greater than ... and specific lysis declined with declining effector : target ratios [13].

Detection of HIV-1 gp120-specific antibody titres

Anti-HIV-1 *env* antibodies in serum were measured using antigen-specific enzyme-linked immunosorbent assays (ELISA). Microtitre plates (96-well; Titertex, ICN Biomedical, Asse, Belgium) were coated with 1 μg/ml $gp120_{W6.1D}$ (MRC) overnight at 4°C. The wells were blocked with phosphate-buffered saline, 0.1% Tween-20, 1% bovine serum albumin for 1 h at 37°C. Serum dilutions were incubated for 1.5 h at 37°C followed by sheep anti-human immunoglobulin–biotin antibodies (Amersham International, Amersham, Buckinghamshire, UK) for 1.5 h at 37°C. Streptavidin–horse radish peroxidase conjugate (Amersham) was incubated for 30 min followed by O-phenyldiamine dihydrochloride substrate (Sigma Chemical Co., St Louis, Missouri, USA). Optical density (OD) was measured at 490 nm. Characterized sera from $gp120_{W6.1D}$-immunized African green monkeys were used as a reference. Anti-gp120 titres were expressed as endpoint dilution titres as compared with standard pooled control serum with a known fixed titre.

Determination of avidity of antibodies

The avidity of ELISA antibody to gp120 of $HIV\text{-}1_{W6.1D}$ was measured by titration of sera in the presence or absence of 35 mmol/l diethylamine. OD values were plotted against $\log_{10}$ serum dilutions. The slope of the curve was calculated to obtain an endpoint titre at an OD of 0.1. The avidity index was calculated as the titre in the presence of diethylamine expressed as a percentage of the titre without diethylamine [14].

Determination of virus neutralization titres

Neutralization assays were performed as previously described [7,15] with minor modifications. In the case of $SHIV_{W6.1D}$, virus was titrated in five replicates from 1 : 25 to 1 : 78 125 on 4×10^4 C8166 cells, pretreated for 60 min with 5 μg/ml Polybrene (Sigma) in RPMI-1640 medium (Gibco BRL, Life Technologies, Breda, The Netherlands) with 10% fetal calf serum (Gibco BRL) and antibiotics in 96-well flat-bottomed plates (Falcon Labware, Becton Dickinson, Oxford, Oxfordshire, UK). The supernatant was changed on days 1, 2 and 3 and twice weekly thereafter. At day 14, supernatant was taken for simian immunodeficiency virus (SIV) p27 antigen determination (Coulter SIV Core Antigen Assay, Coulter Immunology, Miami, Florida, USA). At day 21, cultures were scored for the presence of syncytia. The median infective dose (ID_{50}) of the virus stock was calculated as in previous studies using the Kärber formula [15]. Sera from vaccinated and control animals were heat-inactivated at 56°C for 30 min and serially diluted in duplicate from 1 : 10 to 1 : 160 in ... 75 μl volume. Virus ... was added ... volume. The 96-well plates were ... 37°C, and C8166 cells were subsequently added to ... a final volume of 225 μl. As controls, cultures with C8166 cells only, virus only, and cells and virus without serum were used. The neutralizing titre of a particular serum was defined as the reciprocal of the highest dilution giving a 50% reduction in p27 antigen by ELISA compared with control serum from uninfected animals. To determine whether differences between groups for all immunological assays were statistically significant, we used either the non-parametric Mann–Whitney rank-sum test or the Student's t test [16].

Challenge with chimeric SHIV

$SHIV_{W6.1D}$ [17] was constructed by replacing an *NheI–AvrII* fragment encompassing gp120 and gp41 of the chimeric virus SHIV-4 [8] with the equivalent region of the envelope of the W6.1D molecular clone from virus isolate 320.3 derived from a Dutch AIDS patient [10]. The parental HIV-1 isolate 320.3 was dual-tropic and could infect T cells as well as macrophages [18]. SHIV was passaged four times serially *in vivo*, and PBMC from the last infected rhesus monkey were propagated on autologous rhesus PBMC to finally generate a cell-free stock. The monkey ID_{50} (MID_{50}) was determined by *in vivo* titration in *M. mulatta* [19]. All monkeys were challenged intravenously with 50 MID_{50} of the rhesus PBMC-derived virus stock 4 weeks after the last immunization.

Virology

For quantitative virus isolation and provirus detection by polymerase chain reaction (PCR) before and after challenge, PBMC were purified from blood using lymphocyte separation medium (Organon Technica, Durham, North Carolina, USA). To isolate virus, five-fold serial dilutions of PBMC (starting with four wells of 10^6 PBMC) were cocultivated with 0.5×10^6 C8166 cells per well in duplicate in 24-well plates for 3 weeks. After 3 weeks, cultures were scored for the presence of p24/syncytia to determine the number of virus-producing cells per 10^6 PBMC. For the detection of proviral DNA in PBMC and lymph-node cells, DNA was purified using sodium dodecyl sulphate–proteinase-K digestion followed by ethanol precipitation. Nested PCR was performed using SIV_{gag} and $HIV\text{-}1_{gag}$ primers as described previously [20]. The detection limit of both the SIV_{gag} and $HIV\text{-}1_{gag}$ PCR assay is one copy of proviral DNA per 1.5×10^5 cell equivalents.

Results

Comparison of immunogenicity

In the first experiment, a comparison of the immune responses induced by two different adjuvant formulations

SBAS1 ... SBAS2 ... HIV-1 ... gp120 ... was undertaken (Fig. 1). The SBAS2-W6.1D-vaccinated group (B) had the highest gp120-specific T-helper cell responses throughout the first phase of the study ($P < 0.02$, Student's t-test). In particular, highly elevated numbers of gp120-specific IFN-γ-secreting cells were observed after the third immunization (Fig. 1b). At the same timepoints, antigen-specific IL-2 responses appeared to be low. This was characteristic of IL-2 in this assay when compared with IFN-γ and IL-4 responses, and was probably due to the inherent kinetics of IL-2, which was rapidly consumed by the cultured cells in this assay [12]. This taken into account, the highest IL-2 responses were observed in animals immunized with SBAS2-W6.1D (group B; $P < 0.05$, Mann–Whitney rank-sum test), which peaked 8 weeks after the third immunization. Antigen-specific IL-4 responses were boosted after the third immunization in all animals ($P < 0.01$, Mann–Whitney rank-sum test), but to a higher extent in group B (although this difference did not reach statistical significance) and subsequently declined 6 weeks later. Persistent and high gp120-specific lymphocyte proliferation responses were found in group B, whereas antigen-specific lymphocyte proliferation declined in group A over time ($P < 0.05$, Mann–Whitney rank-sum test, Fig. 1). The highest titrated humoral immune responses as determined by $gp120_{W6.1D}$ ELISA ($P < 0.05$, Student's t test) and $HIV\text{-}1_{W6.1D}$ virus neutralization were measured in group B (Table 1; differences in virus neutralizing antibody titres did not reach statistical significance). In general, antibody responses declined and were boosted following each immunization. T-cell responses after five repeated immunizations (Table 2) differed from those observed after the initial three immunizations performed over the course of the first phase of the study (Fig. 1). Due to delays in preparation of the challenge stock for the second phase (efficacy) of the study, two additional boosts were given. During this delay between experiments, the immune responses matured and the number of antigen-specific IFN-γ-secreting cells dropped off considerably in group B, whereas the number of IL-4-secreting cells was elevated in all animals after repeated boosting.

CTL responses were only occasionally found in some of the animals (Table 2), but were not consistent in each animal over time. CTL responses measured were low (maximal 20% specific lysis; data not shown). Furthermore, due to insufficient samples, the CTL response was not further characterized to be able to determine major histocompatibility complex (MHC) restriction as we observed in earlier studies [13].

Evaluation of HIV-1 vaccine efficacy

In the second phase of the study, we set out to determine which of the vaccines was capable of inducing protective immunity. In addition to groups A and B, a

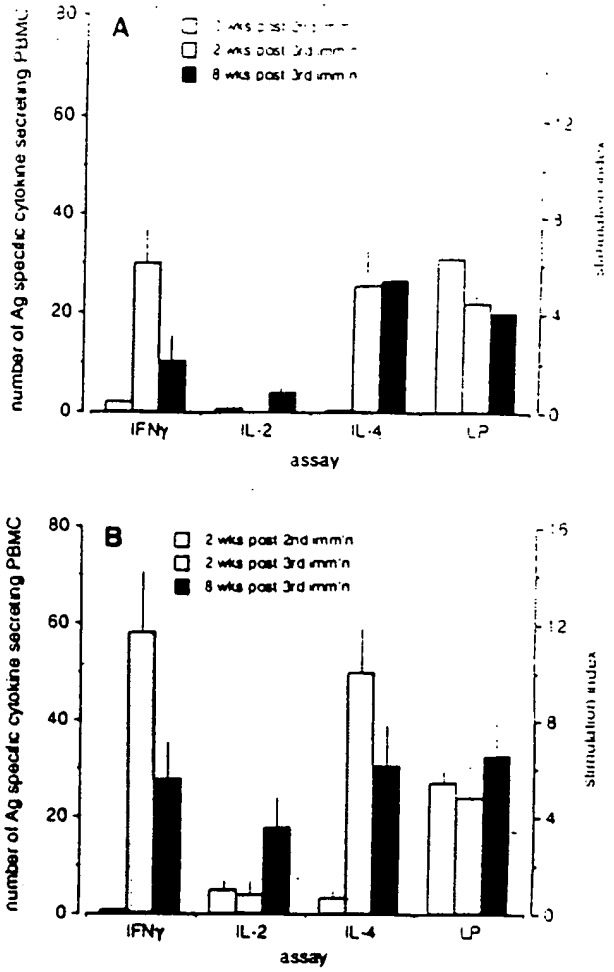

Fig. 1. Comparison of cellular immune responses in *Macaca mulatta* over time. Data are plotted as the mean of four animals ± SEM per group. (a) SBAS1-W6.1D group A ... SBAS2-W6.1D group B. All animals were immunized at ... 4 and 12 weeks with samples assayed at ... and ... weeks. The number of gp120-specific cytokine-secreting cells are expressed per 2×10^5 peripheral blood mononuclear cells (PBMC) for interferon (IFN)-γ and per ... PBMC for interleukin (IL)-4 and IL-2 plotted on the ... axis. Lymphocyte proliferation to gp120 is expressed as the stimulation index (antigen-induced proliferation ... ground proliferation) on the right y-axis. Bars are shown to distinguish between 2 weeks after the second immunization, 2 weeks after the third immunization ... after the third immunization.

third group of four animals (group C) was added ... Materials and methods). A fourth group of four naive animals served as non-immunized controls (group D). One month after the last boost, all animals were challenged with 50 MID_{50} of a rhesus PBMC-propagated, *in vivo* passaged and titrated stock of $SHIV_{...}$ [19].

Post-challenge virology follow-up was performed at 2-week intervals for 3 months with final determination at 4 months post-challenge. All four naive controls

Table 1.

Animal	2 weeks after second immunization: Anti-gp120 titre	2 weeks after second immunization: VNT	Anti-gp120 titre on day of third immunization	[illegible]: Anti-gp120 titre	[illegible]: VNT
Group A (SBAS1-gp120)					
9143	9336	200	< 400	9022	[illegible]
9157	4871	200	< 400	6745	200
9172	7104	100	< 400	8737	[illegible]
9206	6257	100	< 400	8528	200
Group mean ± SEM	6892 ± 936	150 ± 29	< 400	8271 ± 519	300 ± 58
Group B (SBAS2-gp120)					
9150	59310	400	1788	45400	[illegible]
9175	10227	100	616	14216	400
9208	16393	200	1155	21900	[illegible]
9214	5788	100	< 400	9595	200
Group mean ± SEM	22930 ± 12320	200 ± 70	990 ± 310*	22778 ± 7957*	650 ± 150

Virus neutralizing titres (VNT) correspond to the reciprocal of the serum dilution giving 50% protection from cytopathic effect of HIV-1 isolate. *$P < 0.05$, Student's t test.

Table 2. Individual immune responses immediately before challenge*

Animal	VNT $SHIV^{W6.1D}$	Anti-gp120 titres	Avidity percentage	T-helper cytokine† IFN-γ	IL-2	IL-4	Lymphocyte proliferation (SI)	CTL‡ peptide pool
Group A								
9143	20	6400	63	1	0	41	2.8	-
9157	80	12800	80	4	2	91	2.6	A, B, C
9172	80	12800	84	1	0	59	1.5	C
9206	80	12800	89	14	1	58	2.9	B, C
Group B								
9150	320§	25600¶	86	0	HB	74	3.0	B, C
9175	640	51200	82	0	1	82	4.8	-
9208	640	25600	82	0	0	158	15.4	-
9214	320	25600	83	0	1	34	1.5	A, B
Group C								
9171	320	6400	89	0	1	52	5.7	-
9203	20	12800	82	0	0	80	1.5	A
9205	80	25600	85	0	1	63	10.2	-
9241	80	6400	84	0	1	33	0.8	-
Group D								
AA002	< 20	40	-	0	HB	0	0.9	-
1040	< 20	40	-	0	3	0	0.8	-
L146	< 20	< 40	-	0	2	0	0.6	
Y005	< 20	40	-	0	1	0	0.8	

Anti-gp120 titres are expressed as the reciprocal of the endpoint dilution giving more than twice the negative control value. Virus neutralizing titres (VNT) correspond to the reciprocal of the serum dilution giving 50% reduction on the $SHIV_{W6.1D}$ p27 antigen. *Immune responses are from samples taken 2 weeks prior to challenge or on the day of challenge (antibody responses). †gp120-specific cytokine responses above background in the absence of antigen are expressed as the arithmetic mean of cytokine ELIspots per 4×10^5 peripheral blood mononuclear cells (PBMC) for interleukin (IL)-2 and IL-4, and per 2×10^5 PBMC for interferon (IFN)-γ. ‡Cytotoxic T lymphocytes (CTL) based on greater than 10% specific lysis on autologous gp120 peptide-pulsed target cells are represented by the letter of the gp120 peptide pools (7 peptides per pool, overlapping 20-mers) giving positive responses, cumulative over time. §$P < 0.01$, versus group A and C (Mann–Whitney). ¶$P < 0.05$, versus group A (Mann–Whitney). SI, Stimulation index (c.p.m. induced by antigen c.p.m. induced by medium alone). HB, high background that blocked out specific responses.

became infected with a peak virus load at 2 weeks post-challenge, confirming infection (Table 3). In group A (immunized with SBAS1–W6.1D), two out of four animals became infected, but with a delayed infection (a delay in positive PCR signal and virus isolation by 2–4 weeks; Table 3). All animals that received immunizations with SBAS2–W6.1D (groups B and C) remained completely virus-free in PBMC at all time-points tested after challenge. Furthermore, nested PCR and virus isolation on lymph-node biopsies at weeks 2, 4 and 8 post-challenge were all conclusively negative (Table 3). These results confirmed solid protection from infection in 10 out of 12 of the vaccinated animals. From controls and animals not protected, virus was isolated using CCR-5-positive CS1666 cells (confirmed by FACS analysis).

Immune responses and vaccine protection

The immune responses immediately prior to challenge were compared for differences correlating with protection (Table 2). Interestingly, none of the individual

... HIV-1 Env glycoproteins from two series of primary isolates: replication phenotype and immunogenicity. *Virology* 1997, 229:

28 Joag SV, Li Z, Foresman L, *et al.* **Chimeric simian/human immunodeficiency virus that causes progressive loss of CD4+ T cells and AIDS in pig-tailed macaques.** *J Virol* 1996, 70:3189–3197

29 Reimann KA, Li JT, Voss G, *et al.* **An *env* gene derived from a primary human immunodeficiency virus type 1 isolate confers high *in vivo* replicative capacity to a chimeric simian/human immunodeficiency virus** ...

... Reimann KA, ... **Chimeric ... immunodeficiency virus expressing a primary patient human immunodeficiency virus type 1 isolate *env* causes an AIDS-like disease after *in vivo* passage in rhesus monkeys.** 1996, 70:6922–6928

31 Stephens E, Joag SV, Shetter D, *et al.* **Initial characterization of viral sequences from a SHIV-inoculated pig-tailed macaque that developed AIDS.** *J Med Primatol* 1996, 25:

Exhibit 9

08/909,814

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APPLICATION NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO.
08/909,879	08/12/97	PRIEELS	J 04012.0188

EXAMINER

18N2/1124

FINNEGAN HENDERSON FARABOW
GARRETT AND DUNNER
1300 I STREET NW
WASHINGTON DC 20005-3315

ART UNIT	PAPER NUMBER
1818	25

DATE MAILED: 11/24/97

This is a communication from the examiner in charge of your application.
COMMISSIONER OF PATENTS AND TRADEMARKS

OFFICE ACTION SUMMARY

☐ Responsive to communication(s) filed on _____

☐ This action is **FINAL**.

☐ Since this application is in condition for allowance except for formal matters, **prosecution as to the merits is closed** in accordance with the practice under *Ex parte Quayle*, 1935 D.C. 11; 453 O.G. 213.

A shortened statutory period for response to this action is set to expire 3 month(s), or thirty days, whichever is longer, from the mailing date of this communication. Failure to respond within the period for response will cause the application to become abandoned. (35 U.S.C. § 133). Extensions of time may be obtained under the provisions of 37 CFR 1.136(a).

Disposition of Claims

☒ Claim(s) 19-32 is/are pending in the application.
Of the above, claim(s) _____ is/are withdrawn from consideration.
☐ Claim(s) _____ is/are allowed.
☒ Claim(s) 19-32 is/are rejected.
☐ Claim(s) _____ is/are objected to.
☐ Claim(s) _____ are subject to restriction or election requirement.

Application Papers

☐ See the attached Notice of Draftsperson's Patent Drawing Review, PTO-948.
☐ The drawing(s) filed on _____ is/are objected to by the Examiner.
☐ The proposed drawing correction, filed on _____ is ☐ approved ☐ disapproved.
☐ The specification is objected to by the Examiner.
☐ The oath or declaration is objected to by the Examiner.

NOV 26 1997
FINNEGAN, HENDERSON, FARABOW, GARRETT AND DUNNER

Priority under 35 U.S.C. § 119

☒ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d).
☒ All ☐ Some* ☐ None of the CERTIFIED copies of the priority documents have been
☐ received.
☒ received in Application No. (Series Code/Serial Number) 08/356,372.
☐ received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

*Certified copies not received: _____

☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e).

Attachment(s)

☒ Notice of Reference Cited, PTO-892
☐ Information Disclosure Statement(s), PTO-1449, Paper No(s). _____
☐ Interview Summary, PTO-413
☐ Notice of Draftperson's Patent Drawing Review, PTO-948
☐ Notice of Informal Patent Application, PTO-152

Docketed 11/26/97 Attorney DRD/RBR
Case 4012-188-02
Due Date 2/24/98 w/ext
Action Response due
By WHS

--SEE OFFICE ACTION ON THE FOLLOWING PAGES--

VNAS

1. This application is a continuation of serial number 08/442,288 which is now abandoned. The examiner assumes that prosecution is carried over to continuation of the present application since there was no amendment indicating otherwise. In view of this, the office action is directed to the merits of claims 19-32 (see 1062 TMOG, volume 137, 1986).

2. The Group and/or Art Unit location of your application in the PTO has changed. To aid in correlating any papers for this application, all further correspondence regarding this application should be directed to Group Art Unit 1818.

3. The examiner acknowledges the preliminary amendment adding claims 19-32. Claims canceled are claims 1-18.

4. Claims 19-32 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for claims limited to a vaccine composition comprising antigens from Herpes Simplex Virus or the CS protein of plasmodium species or the Hepatitis B surface antigen in combination with adjuvants QS-21 and 3-DMPL and methods of enhancing the immune response, stimulating gamma interferon production and synergistically enhancing the immune response, does not reasonably provide enablement for a vaccine comprising antigens from HIV or FIV. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims.

The claims are drawn to a vaccine composition comprising HIV or FIV antigens and methods. The specification provides no guidance and teaching to enable a vaccine, particularly against HIV. The examiner is interpreting vaccine to indicate protection from disease. The specification provides

no probative evidence to support the claims to a vaccine which would protect humans against AIDS. The obstacles to vaccine development and therapeutic approaches with regard to retroviruses associated with AIDS in humans are well documented in the literature. These obstacles include: 1) the extensive genomic diversity associated with the HIV retrovirus, particularly with respect to the gene encoding the envelope protein, 2) the fact that the modes of viral transmission include virus-infected mononuclear cells, which pass the infecting virus to other cells in a covert form, as well as via free virus transmission, 3) existence of a latent form of the virus, 4) the ability of the retrovirus to "hide" in the central nervous system where blood cells and neutralizing agents carried by the blood cannot reach the retrovirus, due to the blood-brain barrier and 5) the complexity and variation of the elaboration of the disease. The existence of these obstacles establish that the contemporary knowledge in the art would prevent one of ordinary skill in the art from accepting any vaccine or any immunization treatment or any therapeutic regimen on its face. In order to enable claims with regard to drugs and their uses, either in vivo or in vitro data, or a combination of these can be used. However, the data must be such as to convince one of ordinary skill in the art that the proposed claims are sufficiently enabled. When the claims are directed to humans adequate animal data would be acceptable in those instances wherein one of ordinary skill in the art would accept the correlation to humans. Thus in order to rely on animal data there must exist an art-recognized animal model for testing purposes. See In re Hartop, 311 F.2d 249, 135 USPQ 419 (CCPA 1962). With respect to the AIDS-associated retroviruses the art does not recognize any animal model as exhibiting a direct correlation to human disease (see for example Haynes, Science, vol. 260, 1993, pages 1280, copy

enclosed). To date the chimpanzee is the best available animal model for the study of AIDS in humans because it is the only one capable of infection with the HIV or HTLV III/LAV virus. The chimpanzee however, does not develop the full blown syndrome of AIDS, the significance of this failure being the inability to assess challenge after treatment with the purported vaccine. By definition vaccines must not only induce an immune response, but must be immunogenic to the extent that upon subsequent challenge with the live virus, development of the disease is prevented, or better yet infectivity does not occur.

The specification is drawn to enhancing cytolytic responses as well as generating gamma interferon production. However, there appears to be no correlation between these responses and protection from HIV infection. For example, applicant is directed to the study of Butini, et al (already of record) in which it was demonstrated that the existence of high CTL activity in humans with HIV was not predictive of protection or slowing of disease progression. Indeed, Fox has reported (Biotechnology, vol. 12, page 128, 1994, copy enclosed) concerning reports from the First National Conference on Human Retroviruses and Related Infections, that despite some positive results concerning the fight for HIV vaccines and treatment therapies, "AIDS researchers inevitably come back to the conference's central theme. No therapy has emerged as a sure winner in the campaign against HIV, not a preventive vaccine nor a therapeutic vaccine nor any of the immune-system-boosting treatments.".

Additionally, the specification teaches the administration of HSV, CS or Hepatitis antigenic compositions to mice to generate immune responses. The specification fails to provide guidance and

teaching as to doses effective in generating responses to a "feline" virus in mice. It would appear that mice do not become infected with FIV. Therefore, it is not clear how one would reasonably extrapolate from "felines" to mice and it would be difficult to know what doses of these antigens would be effective given a lack of correlation between the two species. In view of all of the above and in view of that which is well known in the art, it is determined that the specification is not commensurate in scope with the claimed subject matter.

5. Papers related to this application may be submitted to Group 1800 by facsimile transmission. Papers should be faxed to Lynette F. Smith, Art Unit 1818 and should be marked "OFFICIAL" for entry into prosecution history or "DRAFT" for consideration by the examiner without entry. The Art Unit 1818 FAX telephone number is (703)-305-7939. FAX machines will be available to receive transmissions 24 hours a day. In compliance with 1096 OG 30, the filing date accorded to each OFFICIAL fax transmission will be determined by the FAX machine's stamped date found on the last page of the transmission, unless that date is a Saturday, Sunday or Federal Holiday with the District of Columbia, in which case the OFFICIAL date of receipt will be the next business day.

6. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Examiner Lynette F. Smith whose telephone number is (703) 308-3909.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Donald E. Adams, can be reached on (703) 308-0570.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the Group receptionist whose telephone number is (703) 308-0196.

SMITH/lfs
November 20, 1997

L. F. Smith

LYNETTE F. SMITH
PRIMARY EXAMINER
GROUP 1800

Exhibit 10

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, DC 20231

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.
[illegible]	[illegible]	[illegible]	[illegible]

FINNEGAN HENDERSON FARABOW
GARRETT AND DUNNER
[illegible] STREET NW
WASHINGTON DC 20005-5315

HM21/0810

EXAMINER
[illegible]

ART UNIT	PAPER NUMBER
1648	

DATE MAILED: [illegible]

RECEIVED
AUG 12 1998
FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner of Patents and Trademarks

Docketed 8/12/98 Attorney DRD/ROR
Case 04012-0182 D2
Due Date 11/09/98 WEEK
Action FINAL RESP. - W/APPEAL
By DRD
BEN

PTO-90C (REV. 2/95)

2-Mail Copy

Office Action Summary

Application No.	Applicant(s)
08/969,879	Priesls et al
Examiner	**Group Art Unit**
Lynette R. F. Smith	1648

☒ Responsive to communication(s) filed on *May 22, 1998*.

☒ This action is **FINAL**.

☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11; 453 O.G. 213.

A shortened statutory period for response to this action is set to expire *three* month(s), or thirty days, whichever is longer, from the mailing date of this communication. Failure to respond within the period for response will cause the application to become abandoned. (35 U.S.C. § 133). Extensions of time may be obtained under the provisions of 37 CFR 1.136(a).

Disposition of Claims

☒ Claim(s) *19-32* is/are pending in the application.

Of the above, claim(s) _____ is/are withdrawn from consideration.

☐ Claim(s) _____ is/are allowed.

☒ Claim(s) *19-32* is/are rejected.

☐ Claim(s) _____ is/are objected to.

☐ Claims _____ are subject to restriction or election requirement.

Application Papers

☐ See the attached Notice of Draftsperson's Patent Drawing Review, PTO-948.

☐ The drawing(s) filed on _____ is/are objected to by the Examiner.

☐ The proposed drawing correction, filed on _____ is ☐ approved ☐ disapproved.

☐ The specification is objected to by the Examiner.

☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. § 119

☒ Acknowledgement is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d).

☒ All ☐ Some* ☐ None of the CERTIFIED copies of the priority documents have been

☐ received.

☒ received in Application No. (Series Code/Serial Number) *08/358,372*.

☐ received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

*Certified copies not received: _____.

☐ Acknowledgement is made of a claim for domestic priority under 35 U.S.C. § 119(e).

Attachment(s)

☐ Notice of References Cited, PTO-892

☐ Information Disclosure Statement(s), PTO-1449, Paper No(s). _____

☐ Interview Summary, PTO-413

☐ Notice of Draftsperson's Patent Drawing Review, PTO-948

☐ Notice of Informal Patent Application, PTO-152

— SEE OFFICE ACTION ON THE FOLLOWING PAGES —

1. The Group and/or Art Unit location of your application in the PTO has changed. To aid in correlating any papers for this application, all further correspondence regarding this application should be directed to Group Art Unit 1648.

2. The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

3. The examiner acknowledges the amendment and the declaration of Dr. Voss.

4. Claims 1-18 have been canceled and claims 19-32 are pending and under consideration.

5. Applicant's arguments filed 5/22/98 have been fully considered but they are not persuasive. The rejection of claims 19-32 under 35 U.S.C. 112 first paragraph, because the specification while being enabling for claims limited to a vaccine composition comprising antigens from Herpes Simplex Virus, CS protein of plasmodia species and Hepatitis B surface antigen in combination with adjuvants QS-21 and 3-DMPL and methods of synergistically enhancing the immune response and stimulating the production of gamma interferon, does not reasonably provide enablement for a vaccine comprising antigens from HIV or FIV, is maintained for reasons set forth in the previous office action.

Applicant urges the declaration of Dr. Voss traverses the rejection because the declaration establishes that the rhesus monkey model is an acceptable model, phase I clinical trials have established the safety and immunogenicity of the vaccine, the invention stimulates a very strong cytokine response and those of ordinary skill in the art would recognize that high CTL levels are indicative of anti-HIV therapy and are the focus of vaccine research.

It is the examiner's position that after review of the information submitted by Dr. Voss, it appears that the following points should be noted. The declaration of Dr. Voss:

a) does not address FIV antigens or vaccines and whether or not the claimed adjuvants are effective in feline immunodeficiency vaccine preparations

b) does not establish the ability of CTL responses to HIV, to reduce viral burden or viral load or that CTL responses generated in response to vaccine administration generated protection

c) does not establish the correlation between HIV-specific CTL responses and slowing of progression to AIDS. Indeed Dr. Voss states that the assays designed to detect HIV-specific CTL responses did not generate any interpretable data.

D) does not establish that a synergistic response was obtained with HIV or FIV and the QS-21 and 3-D MPL adjuvants in the generation of gamma interferon and

e) the claims are drawn to a vaccine comprising HIV or FIV antigens which stimulate gamma interferon and cytolytic T cell responses. The declaration does not establish that T cell responses were generated to HIV and FIV nor the identity of the cell type involved.

In view of all of the information submitted and in view of the specification the rejection is being maintained.

6. **THIS ACTION IS MADE FINAL.** Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO

MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

7. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Examiner Lynette F. Smith whose telephone number is (703) 308-3909.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Donald E. Adams, can be reached on (703) 308-0570.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the receptionist whose telephone number is (703) 308-0196.

SMITH/lfs
August 6, 1998

L. F. Smith

LYNETTE F. SMITH
PRIMARY EXAMINER
GROUP 1800

Exhibit 11

Reprint Series
28 May 1993, Volume 260, pp. 1279–1286

SCIENCE

Scientific and Social Issues of Human Immunodeficiency Virus Vaccine Development

Barton F. Haynes

57. J. Learmont *et al.*, *Lancet* 340, 863 (1992).
58. F. Gao *et al.*, *Nature* 358, 495 (1992).
59. M. T. L. Roos *et al.*, *J. Infect. Dis.* 165, 427 (1992).
60. J. J. Holland, Ed., *Curr. Top. Microbiol. Immunol.* 176, 1 (1992); S. Wain-Hobson, in *The Evolutionary Biology of Viruses*, S. Morse, Ed., in press.
61. A. R. McLean, *Trends Microbiol.* 1, 9 (1993).
62. S. C. Darby *et al.*, *Brit. Med. J.* 298, 1064 (1989); R. J. Biggar, *AIDS* 4, 1059 (1990); G. W. Rutherford *et al.*, *Brit. Med. J.* 301, 1183 (1990); A. Phillips *et al.*, *J. Am. Med. Assoc.* 268, 2662 (1992); A. N. Phillips, C. A. Sabin, M. Befill, G. Janossy, C. A. Lee, *Lancet* 341, 1024 (1993).
63. Although this is a personal view, I am grateful for critical discussions and comments to: B. Åsjo, P. Brown, P. R. Clapham, A. G. Dalgleish, E. M. Fenyo, D. J. Jeffries, M. O. McClure, A. R. McLean, J. P. Moore, F. Miedema, M. Nowak, S. Patterson, A. J. Pinching, H. Schuitemaker, T. F. Schulz, S. Wain-Hobson, and J. N. Weber. Supported by the Medical Research Council.

Scientific and Social Issues of Human Immunodeficiency Virus Vaccine Development

Barton F. Haynes

Development of a preventive immunogen for human immunodeficiency virus (HIV) infection is a national priority. The complexities associated with HIV host-virus interactions, coupled with the rapid progression of the HIV epidemic worldwide, have necessitated lowering expectations for an HIV vaccine that is 100 percent effective and have raised important scientific and nonscientific issues regarding development and use of preventive and therapeutic HIV vaccines.

HIV infection is preventable (*1*, *2*). In spite of this, HIV is spreading worldwide at an alarming rate, and projections of the magnitude of the pandemic by the year 2000 are staggering (*3*). The development of a preventive HIV vaccine (an immunogen administered to HIV-uninfected individuals to prevent infection) is a national priority. Efforts have also begun to develop therapeutic HIV vaccines, whereby HIV-infected individuals would be treated with immunogens designed to boost salutary anti-HIV immune responses, decrease virus-infected cells, and either eradicate HIV or prolong the time until development of acquired immunodeficiency syndrome (AIDS) (*4*–*6*).

HIV Preventive Vaccine Development

The difficult scientific issues before us underlie the fact that, as yet, there is no preventive HIV vaccine on the near horizon with clear prospects for clinical use. What has been developed are (i) promising experimental immunogens and (ii) clear ideas of what the central questions are that should be asked in ongoing and planned human clinical trials (*7*). Whereas traditional non-HIV vaccine development tracks have led to successful killed or attenuated immunogens in spite of lack of knowledge of pathogenic mechanisms or correlates of protective immunity (such as for the development of vaccines for smallpox or polio) (*8*), the emergent nature of the HIV pandemic, coupled with a plethora of critical unknowns, has forced investigators to pursue several vaccine tracks simultaneously in hope of the rapid development of a successful preventive HIV vaccine (*9*, *10*) (Fig. 1).

The author is the Frederic M. Hanes Professor of Medicine at the Duke University School of Medicine and is director of basic research at the Duke Center for AIDS Research, Durham, NC 27710. He serves as co-chair of the National Academy of Sciences Institute of Medicine Roundtable for the Development of Drugs and Vaccines Against AIDS.

Scientific Problems of HIV Preventive Vaccine Development

Although more is known about HIV than almost any other infectious agent, scientific questions remain unanswered that are critical to development of an HIV preventive vaccine.

Optimal requirements for a preventive vaccine. A successful preventive HIV vaccine should be safe and effective for the prevention or quick eradication of initial HIV infection by multiple HIV strains, regardless of HIV exposure by mucosal or parenteral routes (*9*, *11*–*17*). It is important to emphasize, however, that most vaccines prevent disease, not infection. Thus, a successful HIV vaccine may not prevent establishment of infection but still may prevent the development of AIDS. For the

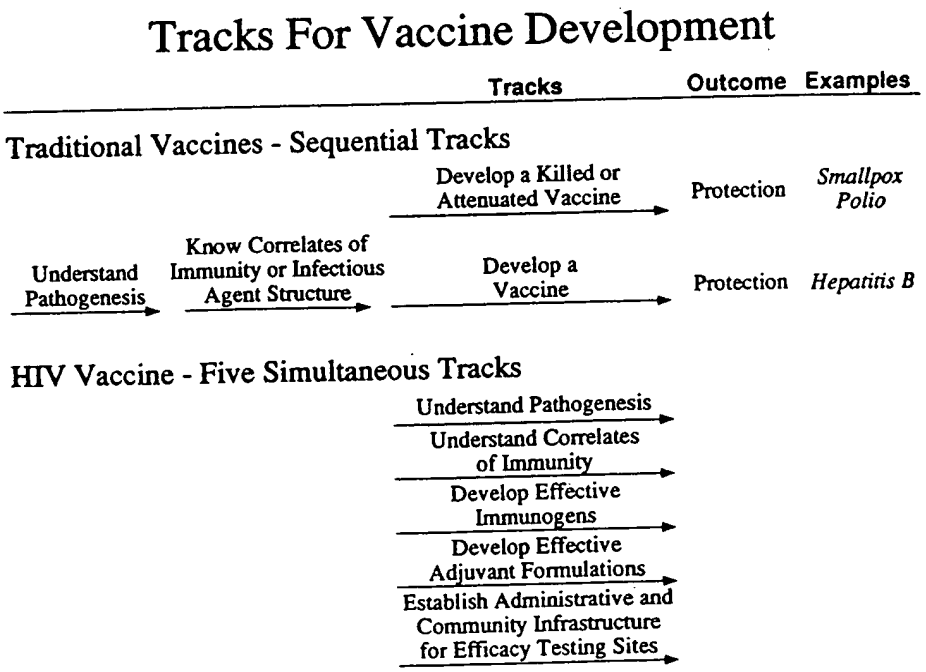

Fig. 1. Approaches to vaccine development. Traditional vaccines either use successful approaches without knowledge of pathogenesis or correlates of immunity (such as with the development of the smallpox and polio vaccines) or proceed in sequential tracks of understanding aspects of pathogenesis, correlates of immunity, or infectious agent structure before development of an effective immunogen (such as with the hepatitis B vaccine). In contrast, HIV vaccine development is proceeding along several simultaneous tracks to maximize the chances of rapidly developing a successful preventive vaccine.

vaccine to be practical, protective anti-HIV immunity should be induced after one or two immunizations, although booster immunizations may be required to provide long-lasting immunity. In children, three to four immunizations may be feasible, as they could be given with other scheduled immunizations. For optimum availability and ease of use, the vaccine should be heat-stable and not require sophisticated measures of preservation. Finally, a successful preventive HIV vaccine should be simple to administer, affordable for all countries, and compatible with other vaccines being administered (*8*, *18*).

Animal models. In spite of an extraordinary amount of work in search of an animal model for human AIDS, no animal model exactly mirrors human HIV infection (*19*). In general, current animal models of HIV or simian immunodeficiency virus (SIV) infection either do not develop AIDS symptoms, do not develop immune responses analogous to human anti-HIV T and B cell responses, or involve the use of endangered species such as chimpanzees (*19*). Thus, many important scientific questions of HIV vaccine development must be answered in human clinical trials.

Correlates of protective immunity against HIV. Because of a lack of an animal model of human AIDS and because a cohort of individuals naturally resistant to HIV infection is not available, the immune correlates of protection against HIV are not known (*9*, *11–17*). For those working on a preventive HIV vaccine, lack of these critical data has forced the design of experimental immunogens that induce some or all of the types of immune responses that are surmised, but not yet known, to be protective against HIV (Table 1). Studies are ongoing to define the types of immune responses that decrease HIV plasma viremia in acute and chronic HIV infection (*20*), that are responsible for the lack of development of AIDS in chimpanzees (*21*), and that are present in HIV seropositive long-term survivors (*22*). In the National Institute of Allergy and Infectious Diseases (NIAID) Multicenter AIDS Cohort Study, HIV seronegative men with recent multiple exposures to HIV, but possibly immune to HIV, have been identified who have T cell (interleukin-2 release) but not B cell (no HIV antibody) responses to HIV proteins (*22*). These data have suggested that cellular immune responses may be protective against HIV infection (*22*).

Pathogenesis of HIV infection. In order to design effective HIV immunogens, researchers must learn about the pathogenesis of HIV. Many investigators have suggested that destruction of the immune system in AIDS is mediated in part by direct pathogenic effects of HIV (*23*) or by HIV-induced immune cell apoptosis, or programmed cell death (*24*). Although neutralizing antibody responses are important for protection against many viral diseases, nonneutralizing HIV envelope antibodies can enhance HIV growth in vitro and might promote progression of HIV infection in vivo (*25*). Molecular mimicry of host proteins such as the major histocompatibility complex (MHC) class I and class II molecules by HIV proteins may be one cause of some or all of the clinical manifestations of AIDS (*26*). Peptides from the HIV gp41 envelope protein suppress immune cell function (*27*) and in some cases induce immunological tolerance to HIV proteins (*28*). Thus, care must be taken that the immunogen selected for an HIV vaccine will induce salutary and not pathogenic immune responses (*29*, *30*).

HIV protein sequence variation. The mutation rate of HIV-1 in HIV-infected patients is estimated to be between 0.1 and 1% per year (*31*, *32*). HIV variability promotes the emergence of neutralization-resistant variants that may be relevant to the persistence of HIV infection (*32*, *33*). Such mutations have been observed in the principal neutralizing determinant [the third variable (V3) region of HIV gp120 envelope protein (*34*)] and at non-V3 loop regions of gp120 as well (*33*, *35*). HIV core protein variants that can escape cytotoxic T cell recognition by similar mechanisms have also been reported to arise in vivo over time (*36*).

This variation means that in every individual there is not just one virus but a swarm of HIV variants, each with different pathogenic properties, growth rates, and varying transmission potential (*33*). Current data suggest that only one variant or group of related variants is passed from host to host, although the factors that determine which HIV variants are passed are not known (*33*). Extensive sequence analyses of DNA of HIV variants worldwide have demonstrated the existence of five subtypes of HIV, with different HIV subtypes found in different geographic locations (*37*). A recent analysis of the eight amino acids at the center of the gp120 V3 neutralizing antibody binding region in 147 variants of the HIV subtype most often present in the United States and Western Europe found 61 unique V3 region sequences in 147 HIV isolates (*37*, *38*). Fortunately, eight sequence motifs accounted for 50% of the HIV isolates analyzed (*38*). An important question is whether it will be feasible to prepare multivalent mixtures of peptides or recombinant proteins that reflect the variable sequences of HIV isolates in particular geographic locations. If an immunogen is to be based on HIV variable sequences, the likelihood for 100% efficacy of a preventive HIV vaccine is small.

A major question is whether it is possible to immunize patients with recombinant envelope proteins that express the conserved (nonvariable), conformation-dependent CD4 binding site and induce broadly reactive neutralizing antibodies that inhibit gp120-CD4 interactions (*39*, *40*). In HIV-infected individuals, the initial neutralizing antibody responses are directed against the viral gp120 neutralizing determinants in the V3 region and neutralize only those HIV isolates with V3 sequences similar to the infecting HIV variant (type-specific antibodies) (*12*, *33*). Broadly reactive neutralizing antibodies arise later that are directed against the site on the gp120 envelope that binds to the HIV receptor on immune cells, the CD4 molecule (*12*, *33*). To date, immunization of HIV seronegative individuals with recombinant envelope proteins has induced primarily type-specific HIV neutralizing antibodies (*40*). A current challenge is to develop new formulations of recombinant envelope proteins that can enhance the induction of broadly reactive HIV neutralizing antibodies.

The need for anti-HIV mucosal immunity. Because a major HIV transmission route is via HIV-infected cells at mucosal surfaces, a successful preventive HIV vaccine should induce both systemic and mucosal protective immunity. Very little is known about the nature of mucosal immunity required

Table 1. Possible correlates of protective immunity for HIV infection.

Immune response	Rationale
HIV neutralizing antibodies	HIV neutralizing antibodies to gp120 protect against an intravenous HIV challenge in vivo (*14*, *42*); neutralizing antibody levels fall as HIV infection progresses (*85*).
$CD8^+$ T cell responses that kill HIV-infected cells or suppress HIV infectivity	$CD8^+$ MHC class I–restricted cytotoxic T cells are important for the control of other viruses such as Epstein-Barr virus, cytomegalovirus, and influenza. Human cytotoxic T cells partially protect SCID-hu mice from HIV challenge in vivo (*86*); $CD8^+$ T cells can inhibit HIV and SIV infectivity in vitro (*30*); cytotoxic T cell activity decreases as HIV infection progresses (*30*).
Anti-HIV T helper cell responses	T helper cell responses are critical for the induction of anti-viral antibodies and for in vivo priming of anti-HIV cytotoxic T cell generation (*87*).

for protection from HIV, whether protection from HIV at mucosal sites is possible at all, or if any systemically administered HIV immunogens induce mucosal immunity. In the SIV model, protection from SIV mucosal challenge has recently been achieved with macaques previously immunized systemically with killed SIV (*41*).

Scientific Problems of HIV Therapeutic Vaccine Development

A hallmark of HIV infection is the persistence of HIV in the host, either in a latent or nonexpressed form, or in peripheral lymph organs in an expressed form (*23*). Treatment of SIV-infected monkeys with killed SIV has resulted in no decrease in viral load and has not delayed the onset of AIDS (*42*). In infected humans, host proteins (recombinant CD4), killed HIV, and recombinant HIV envelope proteins have been used to boost salutary anti-HIV host immune responses (*40*). Although administration of soluble CD4 into HIV-infected seropositive patients was safe, no lasting salutary therapeutic effects were seen (*6*). Immunization of HIV seropositive patients with killed HIV has been safe and in some patients appeared to stabilize immune cell numbers (*6, 43*). The use of the recombinant HIV envelope protein from viral strain LAI ($gp160_{LAI}$) in HIV seropositive patients has also been safe and induced antibody and T cell responses to $gp160_{LAI}$ (*5*). The effect of any experimental HIV immunogen on HIV viral load and CD4 levels in HIV seropositive patients remains to be determined. These early HIV therapeutic vaccine studies, like the early preventive HIV vaccine trials, have used killed HIV or recombinant envelope proteins of the HIV_{LAI} variant—a variant now known to be reflective of only a minority of HIV isolates worldwide (*37*). Prophylactic and therapeutic vaccine trials are now ongoing or planned with envelope proteins from strains MN and SF2 that are more representative of HIV isolates in the United States and Western Europe (*10, 40*).

Many of the immunogens that induce excellent cellular anti-HIV immune responses are live non-HIV vectors (nonpathogenic replicating viral or bacterial agents) containing HIV proteins (*40*). However, the use of live vectors as therapeutic vaccines is not advisable for fear of vector-induced disease, as HIV seropositive patients have compromised immune systems (*44*). Because immunization of animals in-

Table 2. Types of experimental immunogens for HIV vaccine development.

Immunogen	Advantages	Disadvantages or concerns
Live, attenuated HIV strains	SIV with *nef* deleted protects after one immunization; potent inducer of long-lived cellular and humoral immunity; attenuated live HIV could blunt the epidemic by conferring "herd immunity."	Serious concern regarding safety in normal and immunodeficient patients; concern about reversion to virulence; some HIV proteins remaining in the virus may be pathogenic; does not directly deal with variability of HIV strains unless multiple strains of HIV are used.
Inactivated HIV	Simple to prepare; mimics natural infection; inactivated SIV has protected against systemic and rectal SIV challenge.	Host cellular proteins are present in the immunogen; does not deal with variability of HIV strains unless multiple strains of HIV are used.
Protein subunit immunogens (individual HIV proteins such as gp120, gp160, or various types of synthetic peptides of HIV proteins)	Safety, purity; experimental immunogens can be designed that delete potentially pathogenic HIV epitopes; ease of production.	Immune responses to HIV subunit immunogens not long-lasting with current adjuvants; there may not be sufficient immunogenic T cell epitopes on small subunit immunogens to stimulate T cell responses in a cohort of individuals with disparate MHC types.
Multivalent HIV protein subunit immunogen mixtures	Rational strategy for dealing with HIV variability of neutralizing regions of gp120; mixtures can include sufficient T cell epitopes for most participants to respond to, including those with disparate MHC types.	Mixtures of peptides or recombinant gp120 must be based on variable neutralizing domain sequences present in HIV strains in different geographic locations; this necessitates having screening programs to initially define and then follow gp120 neutralizing domain sequences in specific locations.
Subunit immunogens in live vectors (vaccinia, Salmonella, Calmette-Guérin bacillus, poliovirus, rhinovirus, or adenovirus, for example)	Potent inducers of cellular immunity.	Concern for safety in immunocompromised patients; to date, HIV antibody responses induced by these vectors are not good; preexisting immunity to the vector prevents effective boosting by vector.
Anti-idiotypic antibody to CD4 or gp120	May overcome HIV variability problems by inducing broadly neutralizing antibodies.	Induces only antibody responses, not T cell responses; may induce antibodies that interfere with normal CD4 function.
Intracellular immunization (gene therapy)	Would make host $CD4^+$ cells resistant to HIV infection by introducing an HIV resistance gene into $CD4^+$ immune cells.	There are many $CD4^+$ cells in the body of disparate lineages, and the technology is too far underdeveloped to get protective genes in all $CD4^+$ cell lineages; once a protective gene is in cells, resistance may be overcome by HIV mutation; it is not known at present which genes to put in; gene therapy requires isolation of cells from each individual to be treated.
Direct immunization with complementary DNAs of HIV proteins	Promising results in animal protection studies against influenza.	Same concerns as for monovalent and multivalent subunit immunogens; in protection trials of influenza, complementary DNA infection did not protect against injection but protected only against severe disease.
Immunization with host proteins (CD4 or MHC molecules)	Immunogens are nonviral proteins; antibodies to human cellular proteins in SIV grown in human cells protected rhesus monkeys from intravenous SIV challenge, HIV incorporates host MHC proteins when budding from infected cells.	CD4 antibodies theoretically may interfere with CD4–MHC class II interactions and immunosuppress the host; immunization with MHC proteins may make the host resistant to organ transplantation; rhesus monkeys, immunized and challenged with SIV grown in autologous macaque cells, were not protected from SIV infection.

fected with other lentiviruses has, in some cases, led to enhanced disease (45), it is important to continue to carefully monitor the virologic and immunologic sequelae of therapeutic immunizations in future trials in HIV seropositive individuals.

Immunogens for HIV Preventive or Therapeutic Vaccine Development

Table 2 summarizes the types of HIV experimental immunogens currently being tested or being considered for testing in human clinical trials (9–17, 40, 46). Chimpanzees have been protected from HIV and rhesus monkeys from SIV when the challenge virus was given intravenously just at the time of peak neutralizing antibody response from boosting with either killed virus or subunit immunogens (19). The recent successful protection of rhesus monkeys by a single administration of an attenuated SIV strain 2 years before challenge with large amounts of pathogenic SIV has provided the strongest indication to date that a clinically useful preventive HIV immunogen is feasible (47). Although there is concern for the use of an attenuated HIV strain in humans for fear of reversion to virulence and induction of other diseases or conditions such as tumors, the development of an attenuated HIV strain and the demonstration of efficacy for protection against parenteral and mucosal challenge of chimpanzees with multiple HIV strains would provide a benchmark against which other HIV experimental immunogens could be compared. The goal is to design other, less potentially dangerous immunogens that would possess the same efficacy for protection as the attenuated HIV strain.

Currently, most of the immunogens being tested in clinical trials are subunits of HIV envelope proteins, such as gp120 or gp160 (10, 40, 48), or HIV core proteins, such as p17 (10, 40). To address the variable nature of HIV subtypes, researchers have developed mixtures of types of synthetic peptides of neutralizing regions of multiple HIV isolates (40). The ability of HIV and SIV synthetic peptides to prime for $CD8^+$ MHC-restricted cytotoxic T cells with antiviral activity has demonstrated the feasibility of multivalent peptide mixtures as candidates for trials of preventive or therapeutic HIV immunogens (49). However, to date all of the subunit HIV immunogens in animals and in humans induce HIV neutralizing antibodies that last for only several months at most after boosting (40, 48, 50), a feature that would necessitate repeated boosting yearly or more frequently.

Alum (aluminum hydroxide) is currently the only adjuvant formulation approved by the Food and Drug Administration for human use. An essential area of ongoing research is development of new adjuvants that can amplify immune responses to HIV immunogens (51).

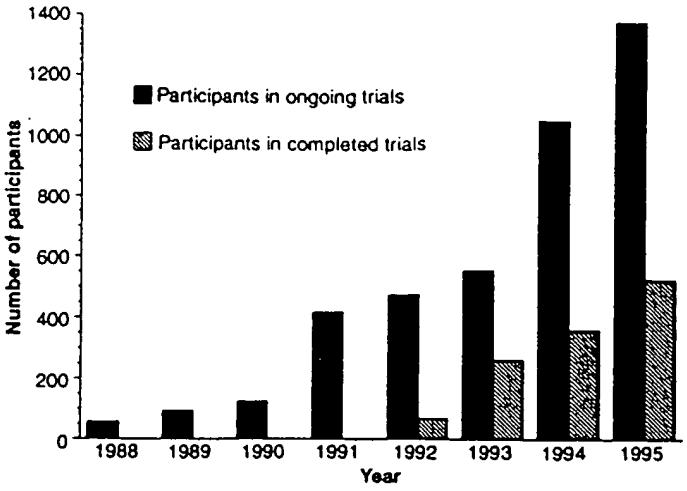

Fig. 2. Phase I and phase II NIAID-sponsored preventive HIV vaccine trials: actual and projected numbers of volunteers. Bars represent cumulative numbers of subjects in ongoing or completed clinical trials. Numbers up to 1992 are actual figures, and numbers after 1992 are projected figures. Source: Division of AIDS, NIAID, NIH.

HIV proteins in live vectors have the potential advantages of an attenuated HIV strain (Table 2) but less of the risk of reversion to virulence (11, 14, 16, 40). Other experimental strategies being considered are (i) immunization with antibodies against the CD4 HIV receptor or gp120 envelope to raise anti-idiotype antibodies that would react with gp120 or CD4 and block HIV infection (52); (ii) intracellular immunization, combining bone marrow transplantation with gene therapy to insert protective genes in immune cells to make cells resistant to HIV (53); and (iii) immunization with complementary DNAs, resulting in the expression of infectious agent proteins (54). This last strategy has resulted in protection of mice and chickens from challenge with influenza and has induced anti-HIV immune responses in mice (54). Finally, experimental immunogens of host proteins such as MHC and CD4 molecules are being considered for the production of an immune response against host molecules involved in HIV infectivity, which would prevent HIV infection (40). In the future, combination of two or more of these immunogen types (one to prime and others to boost) may prove to be superior to single immunogen types for preventive vaccine development. For example, immunogen combinations of live recombinant smallpox and canarypox vectors that express HIV proteins and purified recombinant HIV envelope proteins are being explored (40).

HIV Vaccine Clinical Trials

Phase I trials refer to the first test of a preventive HIV vaccine for safety and immunogenicity in small numbers of low-risk individuals. Phase II trials are additional safety and immunogenicity tests in greater numbers of individuals, with some phase II trials undertaken in high-risk populations. Phase III trials, or efficacy trials, involve large numbers of high-risk individuals; the number of individuals to be tested is determined by the HIV infection rate in the cohort studied, the duration of the follow-up period, the number of participants that do not complete the study, the time needed to achieve maximum protection with the vaccine, and the efficacy rate of the vaccine for prevention of HIV infection (10, 40). A series of phase I clinical trials of recombinant envelope immunogens has been completed (10, 40). A phase II trial including some adults at high risk for HIV infection has begun (55), and phase I trials are about to begin with the use of recombinant envelope proteins as a vaccine for infants born to HIV seropositive mothers (56). It is important to determine if immunization of neonates born to HIV-infected mothers can decrease the incidence of perinatal HIV infection, not only to decrease maternal-child HIV transmission, but also because immunogen efficacy may be easier to define in this setting compared with efficacy trials in adults (56). However, differences in HIV transmission routes between neonates and adults may limit extrapolation of the results of such trials to adults.

There are 16 candidate HIV vaccines in clinical trials in HIV seronegative subjects in the United States, Europe, and Africa, with 8 of these products in the U.S. phase I protocols evaluated by the National Institutes of Health (NIH) (Fig. 2) (10, 40). All together, there are more than 20 candidate HIV vaccines in either preclinical (animal) or phase I or phase II clinical studies (10, 40).

Social and Ethical Issues of Preventive HIV Vaccine Development

The problems of HIV immunology and virology have created a myriad of complex social and ethical issues (46, 57–61). Three

Table 3. Core guidelines for HIV vaccines with regard to future testing for efficacy (*88*). For use in a phase III trial, a candidate vaccine must satisfy condition 1 and at least two of three of conditions 2 through 4.

1. Demonstrated safety in phase I clinical trials.
2. Demonstrated efficacy in HIV-infected chimpanzees or SIV-infected monkeys (with the use of the SIV vaccine analog).
3. Ability to elicit neutralizing antibody that is long-lasting and broadly reactive against heterologous isolates in phase I clinical trials; this would be strengthened by similar induction of long-term and broadly reactive cellular immunity.
4. Demonstrated immunological and genetic similarity to HIV isolates from the proposed efficacy trial study site.

of the more critical areas are ethical design of HIV vaccine clinical trials, community issues related to clinical trials, and issues of clinical trials performed in developing countries.

Design of clinical trials. The design of HIV vaccine phase III efficacy trials has posed major social, ethical, and logistic problems (*10, 40, 62–65*). Although it is beyond the scope of this article to completely review HIV clinical trial design, five major issues of HIV vaccine clinical trials will be highlighted here.

First, the term "vaccine" traditionally signifies safety and protection to many people (*60*). For the reasons mentioned above, it is highly likely that most HIV immunogens will be less than 100% efficacious (*10, 60*). A recent analysis of HIV preventive vaccine efficacy has suggested that earlier use of a 60% effective vaccine would prevent more new HIV infections than later use of a more efficacious vaccine (*10, 66*). Nonetheless, there is a possibility that participation in a phase III efficacy trial could induce more high-risk behavior by creating a false sense of security from the vaccination, thus negating any salutary effect of a partially effective HIV preventive vaccine (*10, 60*).

Any ethical HIV vaccine trial must include counseling to prevent high-risk behavior of vaccinated participants (*59*). Thus, studies need to be performed in the context of HIV vaccine efficacy trials to determine the most effective counseling and education protocols and to study the effect of entrance into the clinical trial on the risk behavior of trial participants (*10, 66, 67*). It is likely that HIV infection rates will fall as a result of counseling and education about how to avoid high-risk behavior (*10, 66, 67*), which could confound the evaluation of the efficacy of the vaccine. In this case, if risk behavior in the face of counseling is carefully monitored, then turning the analysis to look at those individuals who maintained high-risk behavior throughout the trial to evaluate vaccine efficacy might be possible (*10, 67, 68*). Behavioral research is also needed to evaluate incentives provided to enter HIV vaccine efficacy trials in order to prevent coercion of trial volunteers and to prevent giving false impressions of vaccine efficacy (*68*).

Second, immunization with experimental HIV immunogens converts clinical trial participants to varying degrees of seropositivity in HIV antibody tests. Each immunogen tested must have an associated method for distinguishing immunogen-induced seropositivity from HIV infection, and each trial must have a mechanism in place for identification of trial participants to protect insurance eligibility and travel privileges. This problem has been addressed in NIH HIV preventive vaccine trials by issuance of tamper-resistant, numbered identification cards (*10, 60*). However, as the number of HIV preventive vaccine trial participants rises (Fig. 2), protection of uninfected, HIV seropositive vaccine trial participants from discrimination may become more difficult.

Third, the possibility exists that vaccine trial participants will be discriminated against by individuals either afraid that the vaccine itself will cause AIDS or fearful that participation in HIV vaccine trials signifies high-risk behavior (*46, 69*). Although HIV vaccine recipients generally are regarded as altruistic individuals (*46*), strict confidentiality must be guaranteed for all trial participants.

Fourth, considerable debate and concern has been generated over what the entrance criteria should be for phase III efficacy testing of preventive HIV vaccine candidates (*10, 46, 60, 64, 70*). Over the past 1½ years, the Ad Hoc HIV Advisory Panel of NIAID formulated two sets of guidelines for the study of HIV vaccines with regard to future testing for efficacy (*71*). Optimal guidelines for the study of HIV vaccines are essentially the same as the optimal requirements for a successful preventive HIV vaccine. Because no candidate exists as yet that fulfills all of these criteria and because some of the requirements are not yet fully defined (that is, correlates of HIV protective immunity are not known), a second set of core guidelines has been proposed for the entry of experimental immunogens into phase III trials (Table 3). Decisions regarding the selection of individual candidate vaccines for testing in efficacy trials would be made on a case-by-case basis, relative to new information regarding the types of immunity induced by the experimental immunogens and state-of-the-art research on AIDS pathogenesis and clinical correlates of protective anti-HIV immunity (*71*). In light of current gaps in this knowledge, the Ad Hoc HIV Advisory Panel did not believe that sufficient data were available in September 1992 to support selection of HIV preventive vaccine candidates for efficacy trials (*71*). Rather, the panel recommended the formation of the NIAID HIV Vaccine Working Group whose purpose is (i) to lead a coordinated HIV vaccine research effort in the United States among government and nongovernment scientists with the participation of community representatives; (ii) to define critical scientific questions and other issues; and (iii) to help coordinate future studies (*72*).

The core guidelines for HIV vaccine study (Table 3) are the minimum requirements that must be considered before an immunogen is to be taken into efficacy testing. Core criteria may be used to justify entry of an experimental immunogen into a phase III efficacy trial to answer scientific and clinical questions necessary to direct research and future immunogen design (*10*). For example, studies of anti-HIV cytotoxic T cell activity and neutralizing HIV antibodies could be correlated with seroconversion events in a trial to determine the immune correlates of protection against HIV in humans (*10*). Another example of information that could come out of such an efficacy trial would result from genetic study of the HIV isolates from those infected in an otherwise unsuccessful HIV vaccine trial (*10*). If the immunogen tested in the trial was representative of only one HIV subtype and those participants infected during the trial were infected by HIV subtypes other than the subtype represented in the immunogen, then these data would suggest HIV subtype–specific protection and argue for development of a multivalent HIV preventive immunogen (*10*).

Fifth, because there are potential risks of HIV immunogen use (that is, enhancement of HIV infection or induction of autoimmunity) and the true risks of many of the immunogens are not known, obtaining informed consent is difficult (*46, 59, 60, 69*) and the issue of who will provide liability coverage for vaccine-induced injury is a major concern (*61*).

Community involvement in HIV vaccine development. Two central issues are emerging regarding community needs and HIV vaccine trials (*57, 73–76*). First, lack of trust in the U.S. medical establishment has been voiced by both the African American (*73, 74*) and the gay communities (*60, 77*). Both cite multiple reasons for mistrust: lack of government assistance in dealing with the HIV crisis, recent cases of medical fraud, and past examples of unethical scientific behavior, as with the Tuskegee syphilis study (*60, 73, 74, 77*).

Second, there is a need for community involvement in all aspects of HIV clinical trial development efforts. Both NIAID and

the National Institute on Drug Abuse (NIDA) at NIH and the Centers for Disease Control and Prevention (CDC) are collaborating to establish phase III efficacy clinical trial sites in the United States at which there will be ongoing behavioral research (*78, 79*). Educational and counseling objectives will reflect the particular social, ethnic, and political complexities that affect HIV-AIDS research with culturally diverse minority groups (*78, 79*). The goals of the NIAID-NIDA-CDC vaccine preparedness efforts are listed in Table 4 (*78, 79*). Key among these are the initiation of community behavioral research projects and the establishment of community advisory boards to assist in the planning and development of the test sites and test protocols.

Table 4. Goals of the NIAID-NIDA-CDC vaccine preparedness studies. Source: Vaccine Trials and Epidemiology Branch, Division of AIDS, Clinical Research Program, NIAID, NIH, and the Division of HIV-AIDS, CDC (*78, 79*).

1. Development of newly recruited cohorts of individuals at high risk for acquiring HIV infection
2. Measurement of HIV seroincidence among members of these cohorts.
3. Identification of appropriate but noncoercive incentives for recruitment and retention in phase III HIV vaccine trials.
4. Characterization of HIV virus strains in seroincident HIV infections.
5. Determination of recruitment and retention rates among study participants.
6. Development of a rapid risk assessment tool (questionnaire) that is a reliable and valid measure of behaviors that place persons at risk for acquiring HIV infection.
7. Assessment of attitudes toward participation in clinical trials of experimental immunogens with presumed varying levels of efficacy.
8. Development of a standard informed consent form that can be adapted by geographic sites and is known to be understood by volunteers.
9. Determination of the effects of HIV testing, counseling, and trial participation on behaviors that place individuals at risk for acquiring HIV infection.
10. Development of representative and active community advisory boards.

Research teams will need to work with community advisory boards to allay fears that vaccine trials for seronegative subjects might decrease funding and interest in developing immunotherapies for HIV-infected patients and to establish communication and coordinate referrals between HIV vaccine and HIV drug trials (*73, 74, 77–79*). Research teams must also allay fears of government involvement in trials by ensuring that minority participants in clinical trials will be neither excluded nor targeted and establish that the trials are nonexploitive, confidential, and in the best interest of the community (*78, 79*). The New York City Community Vaccine Working Group has outlined principles for community involvement in HIV vaccine trials (*80*). Community advocates already participate in HIV clinical trials planning at both the local and federal levels as members of the NIAID AIDS Clinical Trials Group and the NIAID AIDS Vaccine Evaluation Unit advisory groups. Minority community representatives also serve on the NIAID AIDS Clinical Drug Development Committee, the NIH AIDS Research Advisory Committee, the new NIAID HIV Vaccine Working Group, and the National Academy of Sciences Institute of Medicine Roundtable for the Development of Drugs and Vaccines Against AIDS. Continued involvement of community and patient advisory groups in the HIV vaccine development effort is essential for the HIV vaccine development effort to succeed (*73, 80*).

HIV phase III efficacy trials in developing countries. It is projected that over the next 10 years, the vast majority of new HIV cases will be in developing countries (*3, 81*). The Global Programme on AIDS of the World Health Organization (WHO) has recommended that phase I and phase II trials of HIV candidate vaccines be conducted initially in developed countries, where safety and immunogenicity can be carefully monitored, followed by repeat phase I and phase II trials of some of these vaccines in developing countries where nutritional status and background infections may alter vaccine safety and immunogenicity (*82*). The WHO also recommends that phase III efficacy trials be simultaneously conducted in both industrialized and developing countries, with cohorts with a high incidence of HIV infection (*82*). In addition to the NIH-CDC–sponsored U.S. phase III clinical trial infrastructure, NIAID will also establish HIV vaccine study sites in developing countries and will coordinate their efforts with those of the WHO (*83*). The WHO has selected four countries—Brazil, Rwanda, Thailand, and Uganda—to begin the process of establishing HIV-AIDS vaccine evaluation sites (*82*). The WHO will assist participating countries in providing a favorable environment for national and international collaborative HIV vaccine–related research. In turn, the countries with assistance from the WHO will provide an infrastructure for coordinating national and international collaborative HIV vaccine research (*82*). Work in establishing this infrastructure will include virologic studies to antigenically characterize HIV strains prevalent in the population, epidemiologic studies to quantify HIV incidence in potential groups for future efficacy trials, clinical studies (including repeat phase I and phase II studies of HIV candidate vaccines), and social and behavioral research to develop effective and culturally appropriate methods to educate and counsel vaccine trial volunteers and the general public regarding AIDS and HIV vaccine clinical trials (*82*).

Social and Ethical Issues of Therapeutic HIV Vaccine Trials

Many of the social issues of therapeutic HIV vaccine development are similar to those for preventive HIV vaccine development—community and patient involvement in trial advisory groups, full informed consent, confidentiality, and protection from discrimination for those participating in clinical trials. In addition, there is concern among patient advisory groups that preventive HIV vaccine development efforts will siphon away funds necessary for development of HIV therapeutics (*60, 77*). Clearly, information learned from preventive HIV vaccine trials will greatly assist development of successful therapeutic HIV immunogens by identification of the most potent immunogens and adjuvants. However, sufficient funds must be made available such that efforts to develop both preventive vaccines and therapeutic HIV immunogens can progress unimpeded. It should be emphasized that a far more scientific rationale exists for the feasible development of a preventive HIV vaccine than exists for the development of therapeutic HIV vaccines.

Another critical issue is who should decide what therapeutic HIV immunogens should go forward in clinical trials (*84*). Clearly, for both preventive and therapeutic HIV vaccine trials, rapid evaluation and approval of HIV immunogens by scientific review committees using peer review is essential, and this process should not be bypassed by legislation. The scientific peer-review process will protect patients, study volunteers, academic, industrial, and research communities, and taxpayers.

Conclusions

A lack of answers to key questions regarding HIV vaccine development has led to the need to proceed simultaneously along parallel developmental tracks to answer scientific questions and to establish the infrastructure for a series of clinical trials that will provide for future studies. What is needed now is unprecedented cooperation among U.S. and international academic scientists, government agencies, industry, communities, and patient advocacy groups to establish a comprehensive HIV preven-

tion program, a major component of which is an effort to develop a preventive immunogen for HIV infection (*1*, *2*). The U.S. government should take the lead in ensuring adequate funding for preventive and therapeutic HIV vaccine research, in providing funding for HIV behavioral research, in resolving HIV vaccine liability issues, and in implementing a comprehensive HIV preventive program for all Americans.

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Exhibit 12

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[54] VACCINE COMPOSITION CONTAINING ADJUVANTS

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[57] ABSTRACT

The present invention provides vaccine compositions comprising 3 De-O-acylated monophosphoryl lipid A and QS21. The vaccines compositions are potent inducers of CTL and γ IFN responses.

26 Claims, No Drawings

VACCINE COMPOSITION CONTAINING ADJUVANTS

The present invention relates to novel vaccine formulations, to methods for their production and to their use in medicine. In particular, the present invention relates to vaccines containing QS21, an Hplc purified non-toxic fraction derived from the bark of *Quillaja Saponaria Molina*, and 3 De-O-acylated monophosphoryl lipid A (3 D-MPL).

3 De-O-acylated monophosphoryl lipid A is known from GB2220 211 (Ribi). Chemically it is a mixture of 3-deacylated monophosphoryl lipid A with 4, 5 or 6 acylated chains and is manufactured by Ribi Immunochem Montana.

QS21 is a Hplc purified non-toxic fraction of a saponin from the bark of the South American tree *Quillaja saponaria molina* and a method for its production is disclosed (as QA21) in U.S. Pat. No. 5,057,540.

The present invention is based on the surprising discovery that formulations containing combinations of QS21 and 3 D-MPL synergistically enhance immune responses to a given antigen.

For example, a vaccine formulation of the malarial antigen, RTS, S in combination with 3D-MPL and QS21 results in a powerful synergistic induction of CS protein-specific cytotoxic T lymphocyte (CTL) response in the spleen.

RTS is a hybrid protein comprising substantially all the C-terminal portion of the circumsporozoite (CS) protein of *P. falciparum* linked, via four amino acids of the $preS_2$ portion of Hepatitis B surface antigen, to the surface (S) antigen of hepatitis B virus. Its full structure is disclosed in co-pending International Patent Application No. PCT/EP92/02591, published under Number WO 93/10152 claiming priority from UK patent application No.9124390.7. When expressed in yeast RTS is produced as a lipoprotein particle, and when it is co-expressed with the S antigen from HBV it produces a mixed particle known as RTS,S.

The observation that it is possible to induce strong cytolytic T lymphocyte responses is significant as these responses, have been shown to induce protection against disease in certain animal models.

The present inventors have shown that the combination of the two adjuvants QS21 and 3D-MPL with the recombinant particulate antigen RTS,S results in a powerful induction of CS protein-specific CTL in the spleen. QS21 also enhances induction of CTL on its own, while 3D-MPL does not. The combination can be said to act in a synergistic way, because it has an effect that is larger than the sum of the separate effects of each adjuvant. The synergy between these two adjuvants for CTL induction is a surprising observation which has important implications for the use of recombinant molecules as vaccines for induction of CTL-mediated immunity.

Induction of CTL is easily seen when the target antigen is synthesised intracellularly (e.g. in infections by viruses, intracellular bacteria, or in tumours), because peptides generated by proteolytic breakdown of the antigen can enter the appropriate processing pathway, leading to presentation in association with class I molecules on the cell membrane. However, in general, pre-formed soluble antigen does not reach this processing and presentation pathway, and does not elicit class I restricted CTL. Therefore conventional non-living vaccines, while eliciting antibody and T helper responses, do not generally induce CTL-mediated Immunity. The combination of the two adjuvants QS21 and 3D-MPL can overcome this serious limitation of vaccines

based on recombinant proteins, and induce a wider spectrum of immune responses.

CTLS specific for CS protein have been shown to protect from malaria in mouse model systems (Romero et al. Nature 341:323 (1989)). In human trials where volunteers were immunised using irradiated sporozoites of *P. falciparum*, and shown to be protected against subsequent malaria challenge, induction of CTL specific for CS epitopes was demonstrated Malik et al. Proc. Natl. Acad. Sci. U.S.A. 88:3300 (1991)).

The ability to induce CTLs specific for an antigen administered as a recombinant molecule is relevant to malaia vaccine development, since the use of irradiated sporozoites would be impractical, on the grounds of production and the nature of the immune response.

In addition to malaria vaccines, the ability to induce CTL responses would benefit vaccines against herpes simplex virus, cytomegalovirus, human Immunodeficiency virus, and generally all cases where the pathogen has an intracellular life stage.

Likewise, CTL specific for known tumour antigens could be induced by a combination of a recombinant tumour antigen and the two adjuvants. This would allow the development of anti cancer vaccines.

In certain systems, the combination of 3D-MPL and QS21 have been able to synergistically enhance interferon γ production. The present inventors have demonstrated the synergistic potential of 3D-MPL and QS21 by utilising a herpes simplex antigen known as gD_2t. gD_2t is a soluble truncated glycoprotein D from HSV-2 and is produced in CHO cells according to the methodology Berman et al. Science 222 524–527(1983).

IFN-γ secretion is associated with protective responses against intracellular pathogens, including parasites, bacteria and viruses. Activation of macrophages by IFN-γ enhances intracellular killing of microbes and increases expression of Fc receptors. Direct cytotoxicity may also occur, especially in synergism with lymphotoxin (another product of TH1 cells). IFN-γ is also both an inducer and a product of NK cells, which are major innate effectors of protection. TH1 type responses, either through IFN-γ or other mechanisms, provide preferential help for IgG2a immunoglobulin isotypes.

Glycoprotein D is located on the viral envelope, and is also found in the cytoplasm of infected cells (Eisenberg R. J. et al J. of Virol. 1980 35 428–435). It comprises 393 amino acids including a signal peptide and has a molecular weight of approximately 60 kD. Of all the HSV envelope glycoproteins it is probably the best characterized (Cohen et al. J. Virology 60 157–166). It is known to play a central role in viral attachment to cell membranes in vivo. Moreover, glycoprotein D has been shown to be able to elict neutralizing antibodies in vivo (Eing et al. J. Med Virology 127: 59–65). However, latent HSV-2 virus can still be reactivated and induce recurrence of the disease despite the presence of high neutralizing antibody titre in the patients' sera. It is therefore apparent that the ability to induce neutralizing antibody alone is insufficient to adequately control the disease.

In order to prevent recurrence of the disease, any vaccine will need to stimulate not only neutralizing antibody, but also cellular immunity mediated through T-cells, particularly cytotoxic T-cells.

In this instance the gD_2t is HSV2 glycoprotein D of 308 amino acids which comprises amino acids 1 though 306 of the naturally occurring glycoprotein with the addition of Asparagine and Glutamine at the C terminal end of the

truncated protein. This form of the protein includes the signal peptide which is cleaved to yield a mature 283 amino acid protein. The production of such a protein in Chinese Hamster ovary cells has been described in Genentech's European patent EP-B-139 417.

The mature truncated glycoprotein D (rgD_2t) or equivalent proteins secreted from mammalian cells, is preferably used in the vaccine formulations of the present invention.

The formulations of the present invention are very effective in inducing protective immunity in a genital herpes model in guinea pigs. Even with very low doses of antigen (e.g. as low as 5 μg rgD_2t) the formulations protect guinea pigs against primary infection and also stimulate specific neutralising antibody responses. The inventors, utilising formulation of the present invention, have also demonstrated Effector cell mediated responses of the TH1 type in mice.

Accordingly, the present invention provides a vaccine or pharmaceutical formulation comprising an antigen in conjunction with 3 Deacylated monophosphoryl lipid A and QS21. Such a formulation is suitable for a broad range of monovalent or polyvalent vaccines.

Preferably the vaccine formulations will contain an antigen or antigenic composition capable of eliciting an immune response against a human or animal pathogen, which antigen or antigenic composition is derived from HIV-1, (such as gp120 or gp160), any Feline Immunodeficiency virus, human or animal herpes viruses, such as gD or derivatives thereof or Immediate Early protein such as ICP27 from HSV-1 or HSV-2, cytomegalovirus (esp Human, such as gB or derivatives thereof), Varicella Zoster Virus (such as gpI, II or III), or from a hepatitis virus such as hepatitis B virus for example Hepatitis B Surface antigen or a derivative thereof, hepatitis A virus, hepatitis C virus and hepatitis E virus, or from other viral pathogens, such as Respiratory Syncytial virus, human papilloma virus or Influenza virus, or derived from bacterial pathogens such as Salmonella, Neisseria, Borrelia (for example OspA or OspB or derivatives thereof), or Chlamydia, or Bordetella for example P.69, PT and FHA, or derived from parasites such as plasmodium or Toxoplasma.

The formulations may also contain an anti-tumour antigen and be useful for immunotherapeutically treating cancers.

The formulation may also be useful for utilising with herpetic light particles such as described in International Patent Application No. PCT/GB92/00824 and, International Patent Application No. PCT/GB92/00179.

Derivatives of Hepatitis B Surface antigen are well known in the art and include, inter alia, those $PreS_1$, $PreS_2$ S antigens set forth described in European Patent applications EP-A-414 374; EP-A-0304 578, and EP 198-474.

In a further aspect of the present invention there is provided a vaccine as herein described for use in medicine.

The ratio of QS21:3D-MPL will typically be in the order of 1:10 to 10:1; preferably 1:5 to 5:1 and often substantially 1:1. The preferred range for optimal synergy is 2.5:1 to 1:1 3D-MPL: QS21. Typically for human administration QS21 and 3-D MPL will be present in a vaccine in the range 1 μg–100 μg, preferably 10 μg–50 μg per dose. Often the vaccine will not require any specific carrier and be formulated in an aqueous or other pharmaceutically acceptable buffer. In some cases it may be advantageous that the vaccines of the present invention will further contain alum or be presented in an oil in water emulsion, or other suitable vehicle, such as for example, liposomes, microspheres or encapsulated antigen particles.

Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Md., U.S.A. 1978. Encapsulation within liposomes is described, for example, by Fullerton, U.S. Pat. No. 4,235,877. Conjugation of proteins to macromolecules is disclosed, for example, by Likhite, U.S. Pat. No. 4,372,945 and by Armor et al., U.S. Pat. No. 4,474,757.

The amount of protein in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccinees. Such amount will vary depending upon which specific immunogen is employed and how it is presented. Generally, it is expected that each dose will comprise 1–1000 μg of protein, preferably 2–100 μg, most preferably 4–40 μg. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. Following an initial vaccination, subjects may receive one or several booster immunisation adequately spaced.

The formulations of the present invention maybe used for both prophylatic and therapeutic purposes.

Accordingly in one aspect, the invention provides a method of treatment comprising administering an effective amount of a vaccine of the present invention to a patient.

EXAMPLES

1.0 Synergy between 3D-MPL and QS21 for induction of Interferon γ secretion.

In order to test the ability of 3D-MPL and QS21 based adjuvant formulations of rgD_2t, to induce effector cell mediated immune responses, groups of Balb/c mice were vaccinated, and their draining lymph node cells tested for IFN-γ secretion as described below.

1.1 rgD2t formulations

This experiment compared three adjuvant formulations:

i) rgD_2t in 3D-MPL

ii) rgD_2t in QS21

iii) rgD_2t in 3D-MPL/QS21

These formulations were made up as follows. rgD_2t was produced in CHO cells and corresponds to the mature 1–283 amino acids of HSV-2 gD and is produced according to the methodology of Berman (supra) and EP 0139417.

*rgD_2t/3D-MPL

5 μg of rgD_2t/dose are incubated 1 h, under agitation, at room temperature, then mixed with a 3D-MPL suspension (25 μg/dose). The volume is adjusted to 70 μl/dose using a sodium chloride solution (5M, pH 6.5±0.5) and water for injection to obtain a final concentration of 0.15M sodium chloride, pH is kept at 6.5±0.5.

*rgD_2t/QS21

5 μg rgD_2t/dose are incubated 1 h at room temperature under agitation The volume is adjusted using sodium chloride solution (5M, pH 6.5±0.5) and water for injection to 70 μl. QS21 (10 μg/dose) is then added. pH is kept at 6.5±0.5.and sodium chloride final concentration at 0.15M.

* rgD_2t/3D-MPL/QS21.

5 μg rgD_2t/dose are incubated 1 h at room temperature under agitation. 3D-MPL (25 μl/dose) is added as an aqueous suspension. The final volume of 70 μl is completed by addition of an aqueous solution of QS21 (10 μg/dose) and the pH kept at 6.5±0.5 and the sodium chloride concentration at 0.15M.

1.2 IMMUNISATION

Mice were injected into the hind footpads with 35 μL/footpad of formulation. Thus each mouse received 70 μL. Immunisation were on days 0, and 14. Animals were sacrificed on day 21.

1.3 INTERFERON γ ASSAYS

Popliteal lymph node cells from immnunised mice were stimulated in vitro using rgD_2t at 10, 1, 0.1, 0 μg/ml. Triplicate cultures (200 μl volumes) were set up in round bottom 96-well microtiter plates, using 2×10^5 responder cells and 2×10^5 irradiated (3000 rad) syngeneic naive spleen cells. Culture medium was RPMI 1640 with 10% foetal calf serum. Aliquots of 100 μl of culture medium from each replicate were harvested and pooled for IFN-γdeterminations. Cultures were assayed at 72 hours. For all assays, a control group using ConA (Boehringer Mannheim) at 5 μg/mL was included. This was always positive.

Secretion of IFN-γ was determined using a commercial ELISA assay manufactured by Holland Biotechnology (distributed by Gibco). Assays were carried out on 100 μl of pooled supernatant from triplicate wells.

Secretion of IFN-γ above the assay background of 50 pg/μl was observed in all three formulation groups (see Table). In addition, a synergistic effect between QS21 and 3D-MPL was observed. While each adjuvant on its own induced cells capable of secreting IFN-γ in response to rgD_2t, their combination induced more than twice the sum of individual responses.

1.4 Results

Synergy between QS21 and 3D-MPL for induction of IFN-γ secretion.

Immunization:		QS21/3D-MPL rgD2t	QS21 rgD2t	3D-MPL rgD2t
In vitro	10.0	1351	1105	515
stimulation	1.0	914	116	192
(μg/mL gD2t):	0.1	335	<50	143
	0.0	101	<50	139

IFN-γ is expressed in pg/mL.

The table clearly shows that the combined vaccine induces IFN-γ-secretion in a synergistic manner.

2.0 Synergy Between 3D-MPL and QS21 for the induction of CTLs

In order to test the ability of RTS.S particles in 3D-MPL and QS21 based adjuvant formulations to induce CTLs, groups of B10 .BR mice were immunised and their spleen cells stimulated in vitro and tested in cytotoxicity assays on L cells expressing the CS protein.

2.1 Formulation of RTS.S particles.

RTS.S particles were formulated in three different compositions:

1. RTS.S particles ((10μg) with QS21 (10μg) and 3D-MPL (254μg);
2. RTS.S particles ((10μg) with QS21 (10μg);
3. RTS.S particles ((10μg) with 3D-MPL (25μg);

The formulations were made up as follows:

RTS. S/3 D-MPL

10 μg of RTS.S particles/dose was incubated at room temperature under agitation then mixed with a 3D MPL aqueous suspension (25 μg/dose). The volume is then adjusted to 70 μl/dose using water for injections and a sodium chloride solution (5N, pH 6.5±0.5) to reach a final concentration of 0.15M sodium chloride (pH is kept at 6.5±0.5).

RTS.S/QS21

10 μg of RTS.S particles/dose incubated 1 h. at room temperature under agitation. The volume is adjusted using water for injection and a sodium chloride solution (5N. pH 6.5±05) and completed to a final volume of 70 μ l/ dose with an aqueous solution of QS21 (10μg/dose). pH is kept at 6.5±0.5 and sodium chloride final concentration at 0.15M.

RTS.S /3D-MPL/QS21

10 μg of RTS.S particles/dose are incubated 1 h. at room teperature under agitation then mixed with a 3D-MPL (aqueous suspension (25 μg/dose) The volume is then adjusted with water for injection and a sodium chloride solution (5D pH 6.5±0.5). The final volume is completed by addition of an aqueous solution of QS21 (10μg/dose). pH is kept at 6.5±0.5, and sodium chloride final concentration at 0.15 M.

2.2 Immunisation of mice with RTS.S particles

Four to six week old female mice of the strain B10.BR ($H\text{-}2^k$) were purchased from IFFA CREDO (France). Groups of 3 animals were immunised by intra foot-pad injection of 35 μL of antigen formulation into each hind limb. The animals were boosted with a second equal dose of antigen injected two weeks later.

2.3. In vitro stimulation on anti CS CTL

Two weeks after the boost, spleen cells were harvested and stimulated in vitro using syngeneic fibroblasts transfected with the *P. falciparum* circumsporozoite protein gene (7G8 clone). These CS-transfectant cells have been described in the paper by Kumar, S. et al. (1988), Nature 334:258–260.

The cultures were established in RPMI 1640 medium supplemented with 10% of heat inactivated foetal calf serum and usual additives, in conditions well known to those of skill in the art.

Responder cells were cultured at a concentration of 10^6 cells/mL in the presence of 10^5 CS-transfectants per mL. To prevent proliferation of CS-transfectant cells, these were irradiated using a dose of 2×10^4 rad. The cultures were fed by replacing ½ of culture medium on day 3 and 6, and tested for cytolytic activity on day 7.

2.4. Cytotoxicity assay for anti-CS CTL

Responder cell cultures were harvested, washed, and mixed at ratios varying from 100:1 to 0.3:1 with a constant number of 2000 target cells, in volumes of 200 μL of medium in V-bottom 96-well plates. Target cells were syngeneic fibroblast cells that had been labelled with ^{51}Cr.

Two different types of target cells were used:

1. L cells
2. CS transfected L cells

These are described in: Kumar, S. et al. (1988), Nature 334:258–260.

The assay was incubated for 6 hours at 37° C., then the amount of radioactivity released into the supernatant by lysis of target cells was determined. Cytolytic activity is expressed as % specific lysis:

Results:

		% Specific lysis by formulation:		
Target cells:	Effector: target ratio	1. RTS,S/ QS21/ 3D-MPL	2. RTS,S/ QS21/	3. RTS,S/ 3D-MPL
CS transfected L cells	100	58	17	1
	30	53	10	0
	10	47	5	1
	3	27	1	0
	1	11	0	0
	0.3	2	−2	−1
L cell	100	3	−2	5
	30	−2	1	4
	10	0	−1	2
	3	0	3	4
	1	−1	4	2
	0.3	3	1	2

Immunisation of B10.BR mice with RTS.S adjuvanted with QS21 and 3D-MPL (formulation #1) induced in the

spleen high levels of CTL specific for the circumsporozoite component of RTS.S. Immunisation with RTS.S particles adjuvanted with QS21 (formulation #2) also induced CTL in the spleen, but only at about 1/30 th of the levels given by formulation #1. RTS.S with 3D-MPL (formulation #3) did not induce CTL.

Since the target cells used in this assay do not express MHC class II molecules, the effector cells can be assumed to be $CD8^+$, class I restricted CTL.

3. Other formulation

Hepatitis B Surface Antigen, Alum 3D-MPL and QS21.

The preparation B Surface antigen (HBsAg) is well documented. See for example Harford et al Develop. Biol. Standard 54 p125 (1983), Gregg et al Biotechnology 5 p479 (1987) EP-A-O 226 846 and EP-A-299 108 and references therein. 3D-MPL was obtained from Ribi Immunochem. QS21 was obtained from Cambridge Biotech, and Aluminium hydroxide was obtained from Superfos (Alhydrogel).

A number of different formulations were made up for studies of cell mediated immunity in mice and for studies in Rhesus monkeys.

3.1 Formulation 1 was made up in phosphate buffer (pH 6.8) to comprise the following per 60 µl dose.

20 µg	HBsAg
30 µg	$Al(OH)_3$
30 µg	3D-MPL
10 µg	QS21
10 mM	PO_4^{3-}
0.15M	NaCl

The formulation was made up in the following manner. 20ptg HBsAg/dose was incubated with $Al(OH)_3$ for one hour at room temperature with gentle shaking. 3D-MPL was added as an aqueous suspension, and the formulation completed by the addition of QS21, phosphate buffer and sodium chloride and incubated for one hour at room temperature. The final formulation had a pH of between 6.5 and 7.0 and used for foot pad studies in mice.

3.2 Formulation 2 was made up in a phosphate buffer (pH6.8) to comprise the following per 200 µl dose.

1 µg	HBsAg
100 µg	$Al(OH)_3$
50 µg	3D-MPL
20 µg	QS21
10 mM	PO_4^{3-}
0.15M	NaCl

The formulation was made up in the following manner. HBsAg and $Al(OH_3)$ were incubated together for one hour at room temperature with gentle shaking. The formulation was completed by the addition of $Al(OH)_3$, 3D-MPL as an aqueous suspension and QS21, with phosphate buffer and sodium chloride solution and incubated again for thirty minutes. The pH of the formulation was kept between 6.5 and 7.0 and used for humoral immunity studies in mice.

3.3 Formulation 3 was made up in a similar manner, in a phosphate buffer (pH6.5–7.0) to contain the following per 1 ml dose:

10 µg	HBsAg
500 µg	$Al(OH)_3$
50 µg	3D-MPL
10 µg	QS21

The formulation was used for monkey studies.

4. Conclusions

The combination of the two adjuvants QS21 and 3D-MPL with the recombinant particulate antigen RTS.S resulted in a powerful induction of CS protein specific CTL in the spleen. QS21 enhances induction of CTL on its own, while 3D-MPL does not. The combination can be said to act in a synergistic way, because it has an effect that is larger than the sum of the separate effects of each adjuvant. The synergy between these two adjuvants for CTL induction is a surprising observation which supports our observation of synergy between QS21 and 3D-MPL for induction of T cells capable of secreting IFN-γ in response to stimulation with the soluble recombinant protein rgD_2t. This finding has important implications for the use of recombinant molecules as vaccines for induction of CTL mediated immunity, since the combination of the two adjuvants QS21 and 3D-MPL can overcome this serious limitation of vaccines based on recombinant proteins, and induce a wider spectrum of immune responses than hitherto.

The mouse cell mediated immunogenicity data show that QS21 based formulations of rgD_2t induce a significant synergistic TH 1 type T cell response (IFN-γ secretion).

Such TH1 type T cells have been shown to be involved in induction of delayed type hypersensitivity responses in mice. Our own data in prophylaxis of HSV disease show that concomitant induction of neutralizing antibody titers and antigen specific DTH responses affords the best protection against herpes simplex disease.

Taken together, these data suggested that QS21 formulations of rgD_2t may be effective in inducing a protective response against HSV disease. The data presented show an unexpected synergistic effect between 3D Monophosphoryl lipid A and QS21, in inducing IFN-γ secreting antigen specific T cells. Such a synergy may translate in improved ability to induce a protective response against HSV disease, and indeed these formulations are effective in protecting against disease in guinea pigs.

We claim:

1. A vaccine composition comprising:

(a) an antigen;

(b) QS21; and

(c) 3-De-O-acylated monophosphoryl lipid A (3D-MPL).

2. A vaccine as claimed in claim 1 wherein the ratio of QS21:3D-MPL is from 1:10 to 10:1.

3. A vaccine composition as claimed in claim 1 capable of invoking a cytolytic T cell response in a mammal to the antigen.

4. A vaccine composition as claimed in claim 1 capable of stimulating interferon γ production.

5. A vaccine composition as claimed in claim 2 wherein the ratio of QS21:3D-MPL is from 1:1 to 1:2.5.

6. A vaccine as claimed in claim 1 wherein the antigen is a tumour antigen.

7. A method of treating a mammal suffering from or susceptible to a pathogenic infection comprising the administration of a safe and effective amount of a composition according to claim 1.

8. A method of treating a mammal suffering from cancer comprising the administration of a safe and effective amount of a composition according to claim 1.

9. A process for making a vaccine composition according to claim 1 comprising admixing QS21 and 3D-MPL with an antigen.

10. A vaccine composition as claimed in claim 1 comprising an antigen derived from the group consisting of Herpes Simplex Virus type 1, Herpes Simplex virus type 2.

Human cytomegalovirus, Hepatitis A, B, C or E, Respiratory Syncytial virus, human papilloma virus, Influenza virus, Salmonella, Neisseria, Borrelia, Chlamydia, Bordetella, Plasmodium and Toxoplasma.

11. A pharmaceutical composition useful for adjuvanting an immune response comprising an adjuvanting effective combination of QS21 and 3-De-O-acylated monophosphoryl lipid A (3D-MPL).

12. The composition as claimed in claim 11 capable of invoking a cytolytic T cell response in a mammal to an antigen.

13. The composition as claimed in claim 11 capable of stimulating interferon γ production.

14. A method for stimulating a cytotoxic T cell response in an animal comprising introducing into said animal a cytotoxic T cell response stimulating amount of the composition of claim 1.

15. A method for stimulating a γ-interferon response in an animal comprising introducing into said animal a γ-interferon response stimulating amount of the composition of claim 1.

16. The vaccine composition of claim 1 wherein the QS21 and the 3D-MPL synergistically enhance the immune response in an animal to the antigen.

17. The composition of claim 11 in which the QS21 and 3D-MPL synergistically enhance the immune response in an animal to an antigen.

18. A method of enhancing the immune response in an animal to an antigen which comprises administering to the animal (a) the antigen, (b) QS21, and (c) 3D-MPL.

19. The method of claim 18 in which the animal is a human.

20. The method of claim 18 wherein the QS21 and the 3D-MPL are administered at a ratio of QS21:3D-MPL of from 1:10 to 10:1.

21. The method of claim 18 wherein the ratio of QS21:3D-MPL is from 1:1 to 1:2.5.

22. The method of claim 18 wherein the antigen is derived from any of Herpes Simplex Virus type 1, Herpes Simplex virus type 2, Human cytomegalovirus, Hepatitis A, B, C or E, Respiratory Syncytial virus, human papilloma virus, Influenza virus, Salmonella, Neisseria, Borrelia, Chlamydia, Bordetella, Placsniodium, and Toxoplasma.

23. The method of claim 18 wherein the antigen is a tumor antigen.

24. The method of claim 18 wherein a cytolytic T cell response to the antigen is induced.

25. The method of claim 18 wherein interferon γ production is stimulated.

26. The method of claim 18 wherein the QS21 and 3D-MPL synergistically enhance the immune response.

* * * * *

Exhibit 13

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CAVEAT LECTOR

HERPES SIMPLEX VIRUS: A TOOL FOR NEUROSCIENTISTS

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TABLE OF CONTENTS

1. ABSTRACT

Herpes viruses have received a great deal of attention due to their widespread and ubiquitous prevalence in the human population and to the diverse range of diseases caused as a result of an infection. During the last 20 - 25 years, many research laboratories have investigated the pathogenesis and molecular biology of these viruses; particularly herpes simplex virus (HSV). As a result of this research, HSV has begun to get the attention of neuroscientists. In fact, in the last few years there has been an explosion of research involving the use of HSV and related viruses as tools or model systems for different areas of neuroscience research. This brief review will describe several of these areas including demyelinating diseases, neuronal tracings, and genetic therapy.

2. INTRODUCTION

In order to understand the different roles of HSV in neuroscience, it is necessary to have a general understanding of the different types of viral infections and the replicative cycle.

2.1. HSV Infections

Infection with HSV can result in several diseases ranging from inapparent infections and self-limiting cutaneous lesions to fatal encephalitis (for a review, see 1). In a primary infection, HSV enters the body via mucosal membrane or abraded skin and establishes a local infection in epithelial cells. Viral replication in these cells results in the amplification of virus, the formation of a 'fever blister', and the activation of both cellular and humoral immune responses. During this acute infection, the virus is transported by retrograde axonal transport to the nuclei of the sensory neurons innervating the site of the local infection (2). Studies using animal models have indicated that a limited viral replication occurs within these neurons followed by the establishment of latency.

A latent infection is characterized by the presence of viral genomes (in the nuclei of sensory neurons) and the absence of viral replication or viral protein production (for review, see 3). The infection and establishment of latency within neurons explain why HSV is termed a neurotrophic virus. In a latently-infected neuron, virus-specific proteins are not produced and, as a result, the host's immune system is unaware of the virus' presence and does not target the latently-infected neuron for destruction. Latent infections ensure the survival and persistence of the virus in the human population.

A latent HSV infection is maintained for the life of the host, but the virus can be reactivated periodically to produce infectious virus and recurrent disease. During reactivation, the viral genome in the latently-infected cell is activated resulting in viral replication. The reactivated virus then travels down the sensory axon where it establishes an infection in the epithelia of the skin. Studies using both animal models and human subjects have shown that viral reactivation can be triggered by a variety of stressful or stress-related stimuli including heat, U.V. light, fever, hormonal changes, menses and physical trauma to the neuron (*e.g.* 4-7). While the virus appears to be latent most of the time, HSV infection is probably best characterized by recurrent reactivations and periods of latency.

2.2. HSV Replicative Cycle

HSV is a large, enveloped virus that contains an icosahedral nucleocapsid and a amorphous structure termed the tegument located between the nucleocapsid and envelope. For the purposes of this review, we will briefly review the general replication scheme of HSV (depicted in Figure 1). For a detailed review, the reader is directed to Roizman and Sears (8). The enveloped virus particle binds to the outside of a susceptible cell resulting in a fusion between the viral envelope and cellular membrane. As a result of membrane fusion, the nucleocapsid enters the cell cytoplasm and migrates to the nuclear membrane. The viral genome is released from the capsid structure and enters the nucleus through nuclear pores. Once inside the nucleus, viral-specific transcription, translation, and replication of the DNA genome occur. The newly synthesized viral DNA is packaged into preformed capsid structures and the nucleocapsid buds through the

nuclear membrane, obtaining its envelope. The replication of HSV is fairly quick, occurring within 15 hours post-infection and is extremely lethal to the cell resulting in cell lysis.

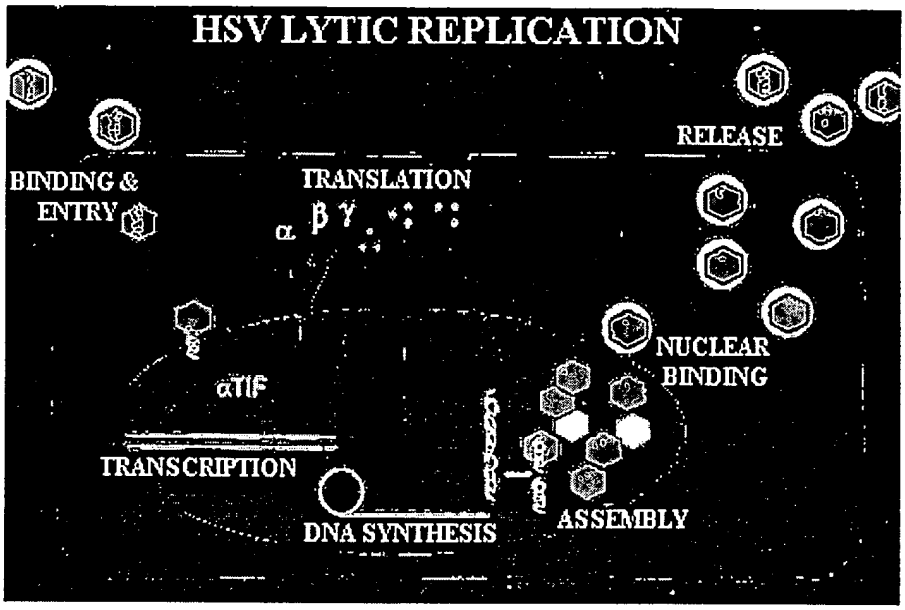

Figure 1. Schematic Representation of an HSV Lytic Replication Cycle.

The genome of HSV is a linear, double-stranded DNA molecule approximately 152 KB in length that encodes for a minimum of 75 separate proteins (9). HSV genes are divided into three temporal classes (alpha, ß, and gamma) which are regulated in a coordinated, cascade fashion (for review see 8). The alpha or immediate-early (IE) genes contain the major transcriptional regulatory proteins and their production is required for the transcription of the ß and gamma gene classes. Of the 5 immediate early genes identified, ICP4 represents the major regulatory protein of HSV. The synthesis of ICP4 is absolutely required for viral replication and this protein is involved in the transactivation of both ß and gamma genes. The ß proteins consist primarily of proteins involved in viral nucleic acid metabolism and are not produced in the absence of alpha proteins. The synthesis of the ß proteins precedes and is required for replication of the viral DNA genome. The gamma proteins consist primarily of virus structural proteins and their synthesis occurs after the onset of viral DNA replication. Molecular studies on a majority of the genes encoded by the HSV genome have demonstrated that many of them can be deleted without interfering with the virus' ability to replicate in cell culture lines (10). In addition, it is possible to construct site-specific mutations, including the deletion of viral genes and the insertion of foreign genes, into the viral genome (11, 12).

The properties of HSV that make it a useful tool for studies in the field of neuroscience include its neurotropism, the ability to construct viral mutants and its ability to establish latent infections in neuronal cells. In this review we will briefly describe several roles for HSV in neuroscience including 1) a model for demyelinating disease, 2) a tool for transneuronal tracing studies, and 3) use as a viral vector for gene therapy.

3. DEMYELINATING DISEASE

3.1. CNS vs PNS Infections

Animal studies have demonstrated that during an acute infection, HSV spreads from peripheral epithelial cells to both the peripheral nervous system (PNS) and central nervous system (CNS; 13-17). Interestingly, cellular damage induced by the virus is markedly different between the PNS and CNS. Tissue damage in the PNS is generally mild while the damage in the CNS is often extensive within a local foci. A well studied example of this concept is seen within the trigeminal root entry zone (TREZ) of the brainstem, a junction region between the PNS and CNS (13, 14, 18). Within this region, following an acute HSV infection, the peripheral myelin is untouched while the CNS side of the trigeminal root develops demyelinated lesions. Within the demyelinated lesions, there is an absence of myelin and the presence of both intact axons and a mononuclear cell (MNC) infiltrate. The exact mechanism of demyelination is not known, but the results from several studies suggest that it is a combination of cellular infection and host immune response.

3.2. Role of Immunosuppression

Glucocorticoid-induced immunosuppression has been reported to reduce immune cell infiltrate and myelin destruction in the CNS portion of the TREZ following peripheral HSV infection (14) and demyelinating lesions were absent in nude (athymic) mice inoculated corneally with HSV (19). A recent study comparing TREZ demyelination following peripheral HSV infection in immunocompetent mice, immune deficient mice, and immunocompetent mice immunosuppressed with glucocorticoids, demonstrated that immune deficient or immunosuppressed mice exhibited reduced levels or no demyelinating lesions (20). These studies demonstrate the role of the host's immune response in demyelination. In addition, other studies have indicated that HSV travels from the site of peripheral infection to the PNS and then to the CNS where it infects and lyses astrocytes. The infection and subsequent lysis of astrocytes occurs prior to the appearance of demyelination (21, 22). Thus HSV-induced demyelination may also be due, in part, to a cytocidal effect of virus replication in astrocytes and oligodendrocytes in the CNS.

The role of immunopathology in HSV-induced CNS demyelination is one reason why HSV infection serves as a useful model for studies investigating human demyelinating diseases such as Bell's Palsy and multiple sclerosis (23). In addition, studies on the mechanism of CNS demyelination and long-term effects of demyelination may serve as good models for trigeminal neuralgia (24).

4. TRANSNEURONAL TRACERS

Neuroscientists have long desired to map chains of neurons in order to identify communication pathways from origin to termination. With the use of antero- and retrograde tracers such as fast blue and horseradish peroxidase it is possible to identify single neurons along with their axons and terminations. However, in order to identify synaptically linked second and third order neurons, transneuronal tracers are required.

4.1. Properties of Effective Transneuronal Tracers

In order for a transneuronal tracer to be effective, it must be specific for synaptically-linked connections, possess the ability to be transported antero- or retrogradely and be sufficiently tagged for efficient and sensitive detection. Substances such as cholera toxin, tetanus toxin and wheat germ agglutinin are known to bind specifically at neuronal membranes and have been used as transneuronal tracers (25-27). These methods, however, have limitations since only small amounts of protein are transported across synapses resulting in an absence of or relatively weak labeling of linked neurons. In addition, nonspecific labeling of adjacent neurons can occur at increased injection concentrations and extended labeling times. Neurotropic viruses, specifically herpesviruses, have an advantage over these other contemporary methods in that they are able to replicate within neuron cell bodies providing signal amplification before infecting second- and third-order neurons. These viruses have also been shown to specifically label neuronal connections in both the retrograde and anterograde direction (28, 29).

4.2. Viral Transneuronal Tracers

The most common transneuronal viral tracers are herpes simplex virus 1 and 2 (HSV-1/HSV-2) and pseudorabies virus (PRV). All three viruses belong to the alpha herpesvirinae family and therefore are neurotrophic (8). The ability of these DNA viruses to specifically infect neurons contributes to their specific transneuronal transport. The most common method used to detect the presence of these viruses in neuronal tissue is by immunohistochemical staining for viral antigen.

Experiments used to obtain transneuronal tracings are modulated by the strain of virus used, the host animal, the site of injection, the amount of virus inoculated, and the time of post-inoculation analysis. The importance of these parameters is apparent from many studies which report that uncontrolled viral tracings, especially at late survival times, may lead to nonspecific labeling (30, 31).

Electron microscopic studies using HSV and PRV have demonstrated that fusion of the viral envelope with the cellular plasma membrane of neuronal extensions is followed by retrograde axonal transport of unenveloped nucleocapsids along axonal microtubules (28). Although this is the primary mode of viral transport to the neuronal nucleus, it is not exclusive. Other studies have shown anterograde transport of virus, (29, 32) and at least one report suggests that the direction of transneuronal transport may be strain dependent (33). By analyzing labeled neurons at progressive time points, it has been determined that retrograde transport occurs much faster than anterograde transport (29). Consideration of the difference in transport rate is important in tracing analyses and can be useful in determining connections between groups of neurons. For example, in groups of neurons which are highly connected by collaterals, one must consider the fact that individual neuron labeling could be due to either antero- or retrograde transport, and in such instances, there may be no way to distinguish between originating and target cells.

Although the release of herpes virus occurs at neuronal terminals, sites of virion egress do not always occur directly into synaptic clefts.

Herpes-containing vesicles have been reported to fuse at presynaptic terminals releasing enveloped virus which then fuses to postsynaptic membranes adjacent to the presynaptic terminals resulting in the entry of nucleocapsids into the neuron. Astrocytes are also susceptible to PRV and HSV infection, but infected cells are only observed subsequent to an adjacent neuronal infection. Ultrastructural analyses of PRV-infected astrocytes have revealed a defect in the cytoplasmic envelopment of viral nucleocapsids rendering the nucleocapsids incapable of plasma membrane fusion. This defect results in an absence of viral egress and an accumulation of virion particles within the cellular cytoplasm (34). The resulting abortive infection effectively prevents astrocytic PRV virions from contributing to nonspecific extracellular spread. At present, no such mechanisms are known for HSV. In fact, several studies have reported that HSV is quite capable of establishing a productive infection in astrocytes (35). The inability of PRV to establish a productive infection in astrocytes provides a great advantage to PRV in ensuring specific transneuronal transport and is a major reason why PRV is considered by many to be the virus of choice for CNS transneuronal tracing studies.

Additional host mechanisms restricting the spread of the virus to non-neuronal cells are provided by the host's immune response to both PRV and HSV infections. Resident microglia, monocytes and macrophages are activated in the nervous system during viral infection and may effectively phagocytose virus and degenerating cellular debris (36). The importance of these mechanisms is apparent considering the large viral load which may be released from necrotic cells to the extracellular space in the absence of these mechanisms. T-lymphocytes may also play a role in the delineation of viral spread (37). Factors regulating these mechanisms have yet to be elucidated, but most likely involve immune-mediated cytokine production and the induction of major histocompatibility antigen expression within the nervous system.

5. GENE THERAPY

5.1 Neuronal Vectors

The advances of modern molecular biology and *in vivo* gene therapy have challenged neuroscientists with the potential prospect of gene manipulation in postmitotic neurons. The ability to alter gene expression in these cells would open the door towards potential therapies for several disorders such as Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis. Gene therapy using viral-based vectors has received considerable attention and represents a major focus of ongoing research in many laboratories. Viral vectors using several different human viruses such as adenoviruses, retroviruses and herpes viruses are currently being developed. Gene therapy directed towards neuronal cells however, presents unique problems. These problems include the genetic manipulation of post-mitotic (*i.e.*, non-dividing) cells, the ability to specifically infect neurons, long-term maintenance of the vector DNA and expression of the target gene within the neuronal cells. Herpesviruses, particularly herpes simplex virus type 1, have unique characteristics of infection, replication and pathogenesis which make them potentially ideal candidates for the development of viral vectors capable of altering endogenous gene expression or delivery of foreign genes both *in vivo* and *in vitro*. The reader is directed to several

reviews on these subjects (38-41).

5.2 HSV-Based Vectors

Herpes viruses have several advantages which lend to their ability to act as neuronal vectors. The HSV genome has been sequenced in its entirety and is rather extensively studied (9). As a result of many years of intense research, a general knowledge exists of which genes and DNA sequences may be deleted and at which sites foreign DNA may be inserted into the DNA genome (10). These studies also have defined the minimal requirements for viral replication and packaging (41). HSV-based vector strategies rely on the ability of HSV to infect neuronal cells and to establish a latent infection. Latency is defined as a state in which viral DNA is maintained within the cell nucleus in the absence of any viral replication. During latency, viral gene expression is largely absent with the exception of the latency-associated transcripts (LAT's) which may remain transcriptionally active (8).

The two main strategies for HSV-based vectors in use today are genetically-engineered viruses and plasmid derived "amplicon" vectors. The first strategy involves the construction of recombinant viruses containing deletions in one or more viral genes whose expression is essential for viral replication (for reviews, see 38-39). These viruses are incapable of producing a productive viral infection (*i.e.*, they are replication incompetent) in normal cells and require a complementing cell line (a cell line that can supply the deleted protein(s) to the virus in *trans*) for replication. Foreign genes can be inserted into theses mutated viral genomes with the goal of producing a virus vector that will infect the target cell (*i.e.*, neurons), and express the foreign gene without killing the cell (due to viral replication). The second strategy involves the use of plasmid derived vectors containing HSV-1 origins of DNA replication and DNA packaging signals which enable multiple copies of the vector genomes to be packaged into helper virus virions (for reviews see 40, 41). Helper viruses can be either recombinant viruses containing a deletion within an essential viral gene or viruses containing temperature-sensitive mutations that prevent replication at 37°C (normal body temperature). In the case of the former, the replication of the helper virus and packaging of the amplicon vector DNA must occur in a cell line capable of complementing the mutations in the helper virus. Plasmid-derived vectors (amplicons) are advantageous because the DNA constructs can be easily manipulated to test endogenous, foreign, antisense or promoter gene expression in the target cell. Although the efficiency of delivery of these multiple copy vectors is high, the primary disadvantage of this system is the fluctuating helper virus to amplicon ratios with passage, which may result in some infected cells not receiving the amplicon genome. Viral titers must be monitored to ensure high amplicon delivery and experimental reproducibility in the absence of wild-type recombinants (41).

Regardless of the vector system used, two primary goals must be achieved to enable long-term gene expression in neuronal cells. The first goal involves the construct of mutant vectors which themselves are noncytotoxic to cells. Several studies have noted active expression of a foreign gene by HSV vector constructs which subsequently became inactivated (41-43). Reasons for this are not completely apparent, but evidence suggests that the inactivation is a result of cytotoxic effects induced by vector systems.

The second goal involves designing stable, active promoters capable of expressing appropriate levels of the foreign protein. The specific promoter involved in individual therapies may change according to the type, status and activity of the neuronal cell of interest. Originally, strong promoter systems such as the human cytomegalovirus IE promoter, the SV40 enhancer, and the RSV LTR were used to drive gene expression. Although such promoter systems were capable of expression they were only active transiently (1 week) and did not result in long-term gene expression (38, 39). Neuronal specific promoters (such as the neurofilament and neuronal-specific enolase promoters) which are believed to be constitutively active in neurons, also produced only transient expression in several HSV vector constructs (38, 39).

During HSV latency the only viral transcripts consistently detected are the latency associated transcripts (LATs). The possibility that the LATs are constitutively expressed in latently infected neurons has made them strong candidates for long-term gene expression in neuronal systems. This hypothesis along with the goal of understanding possible functions and implications of the latent transcripts, has led to a vast literature focused on understanding LAT transcription. The identification of transcriptional activators and suppression mechanisms which may determine functionality in any promoter system is a difficult task considering the modulation which occurs in specific cell types and culture systems. Recently, it has been shown that plasmid derived vectors utilizing HSV-1 promoters are resistant to short-term inactivation and capable of long-term gene expression (44). One possible explanation could be the high copy number of amplicon molecules delivered to individual cells (45). Another explanation stems from data suggesting low level IE gene activity during latency (46). Regardless of the process of sustained activity IE promoters may serve as useful promoter systems in experimental gene transfer vectors.

6. SUMMARY

Herpes simplex virus, as a result of its rather unique life cycle in humans, is a useful tool for many areas of research. Its use in the field of neuroscience represents the newest and certainly one of the most interesting and complex directions of research. Studies utilizing HSV in gene therapy, neuronal tracings, and demyelinating diseases will continue for many years and should provide important insights in areas that up to now have been most difficult to study.

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Exhibit 14

Edition 28

Dorland's *Illustrated* Medical Dictionary

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dermoepidermal junction. Lentigines do not darken on exposure to sunlight, as do freckles. Called also *l. simplex* and *nevus spilus.*

l. malig'na, see under *melanoma.*

nevoid l., a congenital lentigo involving the mucous membranes as well as the skin, occurring in association with various hereditary disorders, including the leopard syndrome and Moynihan's syndrome, and characterized histologically by elongation of rete pegs, an increase in the number of melanocytes with formation of nests, an increase of melanin in both the melanocytes and basal keratinocytes, and melanophages in the upper dermis. Single or multiple lesions may occur, and size and configuration vary widely. Called also *l. simplex* and *nevus spilus.*

senile l., l. seni'lis, a benign, discrete, hyperpigmented macule occurring on chronically sun-exposed skin in adults, especially on the back of the hands and on the forehead. Called also *liver spot* and *solar l.*

l. sim'plex, 1. lentigo. 2 nevoid.

solar l., senile l.

Len·ti·vi·ri·nae (len″tī-vir-i′ne) the HIV-like viruses: a subgenus, *Lentivirus.*

Len·ti·vi·rus (len′tī-vi″rəs) [L. *lentus* slow + *virus*] the HIV-like viruses; a genus of viruses of the subfamily Lentivirinae (family Retroviridae) that cause persistent infection that typically results in chronic, progressive, usually fatal disease. It includes the human immunodeficiency viruses, simian immunodeficiency virus, feline immunodeficiency virus, maedi/visna virus, caprine arthritis-encephalitis virus, and equine infectious anemia virus.

len·ti·vi·rus (len′tī-vi″rəs) any virus of the subfamily Lentivirinae.

len·tu·la (len′chu-lə) lentulo.

len·tu·lo (len′chu-lo, len-too′lo) an engine-driven, flexible, spiral, rotating endodontic instrument made of stainless steel wire, used in a handpiece to place cement into the prepared root canal in root canal therapy. Called also *lentula, lentulo paste carrier,* and *paste carrier.*

Lenz's syndrome (lent′səz) [Widukind D. *Lenz,* German physician, born 1919] see under *syndrome.*

Leo's test (la′ōz) [Hans *Leo,* German physician, 1854–1927] see under *tests.*

le·on·ti·a·sis (le″on-ti′ə-sis) [Gr. *leōn* lion] the leonine facies of lepromatous leprosy, due to nodular invasion of the subcutaneous tissue of the face, giving it a vaguely leonine appearance.

l. os'sea, l. os'sium, bilateral and symmetrical hypertrophy of the bones of the face and cranium, giving it a vaguely leonine appearance; called also *megalocephaly.*

le·o·trop·ic (le″o-trop′ik) [Gr. *laios* left + *tropos* a turning] running spirally from right to left. Cf. *dextrotropic*

lep·rol·o·gist (lep and treatment of

lep·rol·o·gy (lep-r

lep·ro·ma (ləp-ro′ *Mycobacterium le* leprosy. Cf. *lepri*

lep·ro·ma·tous (*romatous leprosy,*

lep·ro·min (lep′ro nodules containi test of immune Called also *Mitsu*

lep·ro·sa·ri·um (treatment and is

lep·ro·sary (lep′r

lep·ro·stat·ic (le *terium leprae.* 2. *leprae.*

lep·ro·sy (lep′rə-s gressive, chroni *prae* and charac neurotrophic les bones, and visce symptoms, cons *lepromatous* type type at the other type, with two s *nomatous.* Called *action,* under *rea* **borderline l.,** an tional between t clinical and hist toward the form downgrading rea **borderline lepro** **borderline tube** **bovine l.,** Johne' **diffuse l. of Luci** **dimorphous l.,** b **indeterminate l.,** ing of a single or hypopigmented macules in expo ously or progres **intermediate l.,** **lazarine l.,** Lucio **lepromatous l.,**

Exhibit 15

Journal of Acquired Immune Deficiency Syndromes and Human Retrovirology

Volume 12, Number 2, June 1, 1996

CONTENTS

(continued on next page)

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Utility of SHIV for Testing HIV-1 Vaccine Candidates in Macaques

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Summary: Intravenous injection of SHIV (simian/human immunodeficiency virus, chimeric virus) into rhesus macaques resulted in a viremia in peripheral blood lymphocytes (PBL) and the generation of anti-HIV-1 (human immunodeficiency virus type 1) envelope immune responses. A challenge stock of a SHIV containing HIV-1 HXBc2 envelope glycoproteins was prepared from infected rhesus monkey peripheral blood mononuclear cells (PBMC). The minimum animal infectious dose of the SHIV stock was determined and used in a challenge experiment to test protection. The vaccination of two rhesus monkeys with whole inactivated HIV-1 plus polydicarboxylatophenoxy phosphazene (PCPP) as the adjuvant protected the animals from becoming infected by a SHIV challenge. This experiment demonstrated for the first time that monkeys immunized with HIV-1 antigens can be protected against an HIV-1 envelope-containing virus. As the challenge virus was prepared from monkey PBMC, human antigens were unlikely to be involved in the protection. Protection of rhesus monkeys from SHIV challenge may help define protective immune responses stimulated by HIV-1 vaccine candidates. Key Words: Simian/human immunodeficiency virus chimera—Rhesus monkeys—HIV-1 vaccine.

One of the obstacles to HIV-1 vaccine development is the lack of an applicable animal model. The only nonhuman host in which HIV-1 can efficiently replicate is a chimpanzee. However, the cost and availability of this endangered species make it almost impossible to do sufficient experimentation needed to test vaccine efficacy. Although SIV (simian immunodeficiency virus) causes an AIDS-like disease in monkeys and its genetic organization shares remarkable similarity with HIV-1, vaccine studies have indicated that the immune reactive epitopes of the SIV envelope glycoprotein are different from those in its HIV-1 counterpart (1). This critical difference in the major protective antigen may diminish the predictive value of SIV as a model for HIV-1 vaccine candidates.

To overcome this problem, a molecular hybrid of SIV and HIV-1, designated SHIV, was engineered by replacing the *env*, *tat*, and *rev* genes of SIVmac239 with their respective HIV-1 HXBc2 counterparts. The SHIV-HXB construct used in this study also contains the HIV-1 *vpu* gene, which is

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absent in all known SIV genomes and whose expression in SHIV appears to enhance viral replication in vivo (20). This hybrid virus combines SIV's replicative ability in macaques monkeys together with the ability to encode HIV-1 envelope proteins. It has been shown that SHIV replicated in macaques cynomologus with a detectable viremia that lasted at least 3 months. All SHIV-infected monkeys had antibodies against the HIV-1 envelope glycoprotein, and many animals also produced antibodies reactive with the SIV gag proteins (2). These observations indicate the SHIV infection of macaques may serve as a valid animal model for assessing the efficacy of HIV-1 envelope-based vaccines.

MATERIALS AND METHODS

Preparation of the SHIV Challenge Stock

Approximately 100 ml of venous blood was collected from eight rhesus monkeys housed at the New England Regional Primate Research Center. Peripheral blood mononuclear cells (PBMC) were isolated by histopaque layer centrifugation and stimulated in culture by Concanavalin A (Con-A). The cells were then infected with SHIV-HXB that was prepared from infected CEM×174 cells and contained an reverse transcriptase (RT) activity of 10,000 cpm (2). The infected monkey PBMC were cultured in RPMI 1640 containing 10% fetal calf serum (FCS) and 20 U/ml recombinant human IL-2 for 5 days. The supernatant of the infected monkey PBMC was collected at days 5 and 6 postinfection (p.i.) and was filtered through a 0.45-μm filter unit. This culture supernatant consisted of an ~800 ml volume and was aliquotted into 1-ml cryovials and stored at −80°C. A number of the frozen vials were tested for $TCID_{50}$ (median tissue culture infectious dose) using CEM×174 cells. The radiolabeled cell lysates were precipitated with pooled sera from HIV-1–infected individuals to document the hybrid nature of the SHIV virus. Proteins corresponding to the HIV-1 *env* and *vpu* products and to the SIVmac *gag/pol* products were identified (data not shown).

Detection of SHIV Replication in *Macaca mulatta*

The infectivity of the SHIV challenge stock was tested in rhesus monkeys by means of intravenous injection. Approximately 10 ml of heparinized blood was collected from each inoculated animal at various times p.i.. PBMC were isolated by histopaque layer centrifugation and washed with phosphate-buffered saline (PBS). Approximately 4×10^6 monkey PBMC were co-cultivated with 2×10^6 CEM×174 cells in RPMI 1640 media containing 10% FCS. The mixed cells were stimulated with Con-A (5 μg/ml) overnight and were then cultured in RPMI 1640 medium containing 20 U/ml human IL-2. The detection of SIV gag p27 released into the culture medium by the SHIV infected cells was assayed a total of three times at 2-, 3-, and 4-week intervals following co-cultivation using Coulter's SIV core antigen detection kit (Coulter, Hialeah, FL, U.S.A.). Virus detection was scored as positive when p27 was present in the culture medium in at least two of the three time points. Virus load in the PBMC was determined by limiting dilution co-culture. In brief, 12 serial 1:3 dilutions of PBMC beginning with 1×10^6 cells were co-cultured in duplicate with 1×10^5 CEM×174 cells per well in 24 well plates (total volume, 1 ml). After 3–4 days of culture, 1 ml of RPMI 1640 media was added to each well. The culture was then split every 3 days at a 1:1 dilution. Supernatant samples were collected after 21 days of culture and assayed for SIV p27 antigen. Virus load was calculated as the number of PBMC present in the dilution at which 50% of the co-cultures were infected as determined by the p27 assay.

Serum was separated from clotted blood samples by low-speed centrifugation. The presence of anti-SHIV antibodies in the serum was tested by Western analysis using Dupont HIV-1 strips. $CD4^+$ T-cells were measured as described previously by Daniel et al. (3).

HIV-1 gp120 Antibody ELISA, Neutralizing Antibody Assay, and PCR Analysis

Recombinant HIV-1 gp120 (HXBc2 strain) was produced from a baculovirus expression system and was purified by either lentil lectin or immunoaffinity columns according to the protocol described by Gilljam (4). ELISA plates were coated with 400 ng per well (Nunc-Immuno Plate Maxisorp, 96 wells). The secondary antibody was a rabbit anti-monkey IgG peroxidase conjugate (Sigma) used at a 1:40,000 dilution. The titer of anti-HIV-1 gp120 is defined as the highest dilution of serum that produces twice the optical density (OD) value of the negative control at that dilution.

The presence of neutralizing antibodies against HIV-1 in the plasma samples was detected by an antigen release assay. In brief, SHIV-HXB virus containing 40 $TCID_{50}$ units was incubated in triplicate with plasma samples at a 1:2 dilution. Plasma samples from an HIV-1–infected patient (1:12) and an uninfected rhesus monkey served as positive and negative controls, respectively, for neutralizing activity. Following 30 min of incubation at room temperature, the samples were mixed with 50,000 CEM×174 cells. The cells were maintained in RPMI 1640 medium containing 10% FCS for 4 days, with fresh medium being added every 2 days. The SIV p27 antigen released into the culture supernatant was measured by the p27 assay, and the average of the three duplicates was determined. As shown in Fig. 3 below, a culture with no plasma present provided an average of 480 pg/ml p27 antigen, whereas cultures without SHIV gave an average of 18 pg/ml antigen as the background values for the assay.

The presence of SHIV in the plasma of immunized/challenged monkeys was analyzed by competitive RT-PCR. A competitor synthetic RNA was generated as follows. A 711–base pair (bp) DNA fragment from SIVmac239 *gag/pol* genes (SIVMM239 nucleotide sequence 2256-2966) (5) was cloned into the pCRII vector. A 60-bp bacterial phage DNA was inserted into a *Stu* I site in the 711-bp SIV *gag/pol* fragment, generating plasmid pEX1-11. The competitor RNA was synthesized from this modified SIV *gag/pol* sequence by in vitro transcription using Sp6 RNA polymerase. Viral particles were pelleted from 1-ml plasma samples by centrifugation at 14,000 rpm for 1 h, from which viral RNA was extracted using the Micro-scale Total RNA Separator Kit (Clontech). Reverse transcription of the viral RNA was carried out in the presence of 0 or 10^4 molecules of the competitor RNA, 5 μ*M* of random hexamer primer (Pharmacia), RNase in-

hibitor, and M-MLV RT (Gibco BRL) at 37°C for 90 min. The cDNA was subsequently used for PCR amplification using a 5' primer (5'ATGGCCAAATGCCCAGACAGAC, SIVMM239 nucleotide sequence 2578) (5) and a 3' primer (5'AGAGAGAATTGAGGTGCAGCA, SIVMM239 nucleotide sequence 2824) (5). A 247-bp fragment was amplified from the SIV viral cDNA, whereas a larger fragment (310 bp) was amplified from the competitor cDNA. PCR samples were analyzed on a 2.5% agarose gel and confirmed by Southern blot using the SIV *gag*-specific DNA probes (data not shown).

RESULTS

Rhesus macaques (*Macaca mulatta*) are more readily available than cynomologous macaques (*Macaca fascicularis*), and a great deal of data has accrued from the use of rhesus monkeys in human vaccine and drug studies. To develop this animal model, SHIV-HXB virus was prepared from infected rhesus monkey PBMC on a large scale, and its infectivity was then determined by $TCID_{50}$ titration in CEM×174 cells. Two rhesus monkeys were intravenously injected with 10,000 $TCID_{50}$ units. As shown in Table 1, SHIV-HXB replicated efficiently in rhesus monkeys as measured by virus isolation from the PBL of the infected animals but has no apparent effect on CD4 cell counts. These observations agree with previously published data showing that the peripheral blood of cynomologous monkeys was permissive for SHIV replication but that no significant effect on the number of $CD4^+$ T cells of the infected animals was seen (2). This data indicates that rhesus macaques may be a useful animal model for the study of SHIV infection.

The minimum amount of virus required for establishing productive replication in rhesus monkeys was determined by intravenous injection of four animals with 4000, 400, 40, or 4 $TCID_{50}$ units, respectively. Table 2 shows that all the animals became infected and that the level of virus load in the peripheral blood was independent of the size of the virus inoculum. In a second experiment, four rhesus monkeys were intravenously injected with 4, 0.4, 0.04, or 0.004 $TCID_{50}$ units, respectively. Table 2 shows that only the animal inoculated with 4 $TCID_{50}$ became infected. On the basis of this data, an intravenous injection dose containing 24 $TCID_{50}$ should have a 99% probability of infecting a rhesus monkey, whereas an injection of 5.6 $TCID_{50}$ of this virus should have a 90% infection probability (6). All the infected animals in Tables 1 and 2 developed serum antibodies against both HIV-1 gp120 and SIV antigens. Cell-mediated immune responses specific to HIV-1 gp120 were also detected in the infected animals (N. Letvin, unpublished observations). Nonetheless, all the animals appeared healthy despite the established virus infection.

TABLE 1. *Detection of SHIV-HXB in PBMCs of* Macaca mulatta *(Mm, rhesus monkeys) and the* $CD4^+$ *T-cell count*

Week	Mm421 CD4 count	Mm421 virus isolation	Mm337 CD4 count	Mm337 virus isolation
0	1,860	–	396	–
3	1,277	+	637	+
5	819	+	485	+
10	2,176	+	547	+
15	1,802	–	837	–
19	1,320	+	936	+
22	3,374	–	1,411	–
26	2,892	+	1,137	+
30	2,127	–	1,083	+
35	2,085	+	1,133	–
40	2,264	–	946	–
43	2,911	–	816	–
56	3,542	+	1,357	+

Vaccine development can benefit from the availability of correlates of immunity as elucidated in an animal model. Attenuation of the viremia observed in SHIV-infected rhesus macaques might be one predicted consequence of a protective immune response. Currently, most efforts toward determining the appropriate HIV-1 protective antigen have centered on nonreplicating antigens (7), which quite often require the addition of an adjuvant to potentiate their otherwise poor immunogenicity. The ideal combination of antigen and adjuvant are conformed so that important epitopes are maintained and an appropriate immune response is engendered. We have recently described a water-soluble high molecular weight polyelectrolyte phosphazene species (polydicarboxylatophenoxy phosphazene; PCPP) that has remarkable adjuvant properties (8,9). PCPP has facilitated the induction of very high functional antibody titers against several antigens.

The utility of SHIV in testing the efficacy of HIV-1 vaccine candidates was initiated by immunization and challenge of four rhesus monkeys. The animals were immunized intramuscularly with the following 1-ml formulations: animal Mm91069 received 100 μg recombinant HIV-1 SF2 gp120 produced from Chinese hamster ovary cells with 100 μg polyphosphazene (PCPP) as adjuvant; animal Mm91080 received 100 μg formalin-inactivated HIV-1 LAI virus produced from infected CEM×174 cells; and animals Mm91083 and Mm91084 both received 100 μg inactivated HIV-1 LAI virus plus PCPP.

TABLE 2. *Detection of SHIV-HXB in PBMC of* Macaca mulatta *infected with different doses*[a]

Week	MML3 4600 $TCID_{50}$	MML28 460 $TCID_{50}$	MML9 46 $TCID_{50}$	MMJO28 4 $TCID_{50}$	MM8A2 4 $TCID_{50}$	MMS-88 0.4 $TCID_{50}$	MM8B7 0.04 $TCID_{50}$	MME363 0.004 $TCID_{50}$
2	460	51	910	100	6	ND	ND	ND
3	4,100	8,300	37,000	1,400	460	ND	ND	ND
4	37,000	2,700	37,000	8,300	910	ND	ND	ND
6	280,000	74,000	670,000	280,000	25,000	ND	ND	ND
8	110,000	110,000	670,000	330,000	8,200	ND	ND	ND

[a] The numbers are the calculated PBMC in the dilution at which 50% of the co-cultures became infected as determined by the assay for SIV p27 antigen. The lower the number of PBMC required to establish infection, the higher the virus load in the blood of the infected animals.

By week 5 after the first immunization, only the two animals that received both whole inactivated virus plus PCPP developed serum antibodies against HIV-1 gp120 as measured by either ELISA or Western analysis (Figs. 1 and 2). The anti-gp120 antibody levels of all four animals had fallen below the level of detection by ELISA by week 43 (Fig. 1), and therefore all the animals received a secondary immunization at this time with 100 μg recombinant HIV-1 HXBc2 gp120 produced by a baculovirus expression system plus PCPP. The choice of antigen for the boost was due merely to the unavailability of HIV-1 SF2 gp120 and whole inactivated HIV-1 LAI. The boost significantly increased the level of serum anti-gp120 antibodies in all the animals. Animals immunized with inactivated HIV-1 virus plus PCPP (Mm91083 and Mm91084) had the highest titers, which were significantly higher than those seen in the other two animals. The antibody response in the vaccinees was confirmed by Western blot analysis using a commercial HIV-1 diagnostic kit. As shown in Fig. 2, the two animals that received inactivated virus plus PCPP developed antibodies against HIV-1 gag proteins that were detectable by week 5 and persisted until week 43. The second immunization merely increased their response to gp120. The adjuvant effect of PCPP on the immunogenicity of HIV-1 antigens appeared to be significant.

A third immunization identical to the formulation as in the second immunization was administrated to all the animals at week 73. Two weeks later (week 75), all the vaccinees were challenged intravenously with 24 $TCID_{50}$ units of SHIV-HBX, which was previously calculated to have a 99% probability of establishing infection. None of the four animals had significantly neutralizing activity in their plasma against SHIV infection at the time of SHIV challenge (Fig. 3). Blood samples were collected from the animals at 3-week intervals after the SHIV challenge and analyzed for SHIV replication by peripheral blood co-culture with human T cells (Table 3). Viruses were isolated from the PBL of animal Mm91069 in the first 2 weeks following SHIV injection, and from the PBL of animal Mm91080 became infected between weeks 2 and 5 after the injection of the SHIV challenge virus. The other two animals, Mm91083 and Mm91084, remained virus isolation negative throughout the experiment (week 24 postchallenge). PCR analysis of the blood samples measuring viral RNA in the plasma (Table 4) confirmed the virus isolation data. Western blot analysis of serum samples from the challenged animals compared sera taken from the animals before and after the SHIV challenge (Fig. 4). The protected animals, Mm91083 and Mm91084, showed no detectable changes in their antibody profile. The unprotected animals, Mm91069 and Mm91080, developed antibodies against HIV-1 gp41 monomer and tetramers, as well as antibodies against the p24 Gag protein (Fig. 4). Because the four animals differed only in the formulation used in the primary immunization, it is important to note that the two protected animals were both immunized with inactivated HIV-1 plus PCPP (Mm91083 and Mm91084). Whether this was due to PCPP as the chosen adjuvant requires further study, as the unprotected Mm91080 lacked any comparable adjuvant in the primary immunization. It is also noted that animal Mm91069 received recombinant gp120 from different strains of HIV-1 in the primary and both boost immunizations, which may have resulted in lack of protection. Nonetheless, by showing evidence of protection this experiment demonstrated that the aforementioned monkey model can be used to test HIV-1 vaccine candidates.

DISCUSSION

In the past 10 years, several HIV-1 vaccine candidates have been tested in chimpanzees. Some

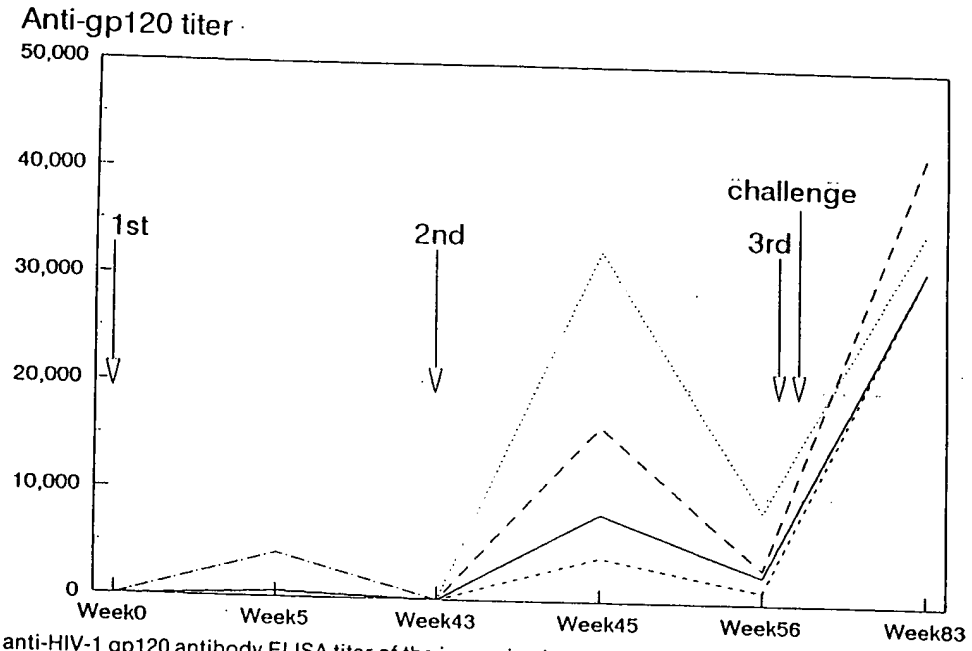

FIG. 1. Serum anti-HIV-1 gp120 antibody ELISA titer of the immunized monkeys. Titer is defined as the highest dilution of serum sample which gives twice the value of the negative control OD at that dilution. Mm91069, 100μg HIV-1 SF2 gp120+PCPP; Mm91080, 100 μg HIV-1 LAI whole inactivated virus; Mm91083 and Mm91084, 100 μg HIV-1 LAI whole inactivated virus+PCPP. All four animals were boosted twice with 100 μg HIV-1 HXBc2 gp120+PCPP and challenged with 24 TCID50 of live SHIV-HXB by intravenous injection. The first immunization occurred at week 0. The second immunization was administered at week 43. The third immunization was given at week 71, and the SHIV-HXB2 challenge was administered at week 73 after the first immunization. Mm91069, solid line; Mm91080, short dashed line; Mm91083, dotted line; Mm91084, long dashed line.

have produced promising results (10–12). From the vaccine development point of view, further studies should test optimal vaccine formulations, including a comparison of different adjuvants, the antigen doses and the optimal schedule of immunization to stimulate maximal protection against challenge by different HIV-1 strains. However, it is almost impossible to conduct these necessary studies in statistically significant numbers of chimpanzees. According to a report from the AIDS Vaccine Surveillance System (13), the total number of chimpanzees used in HIV-1 vaccine challenge studies by 1992

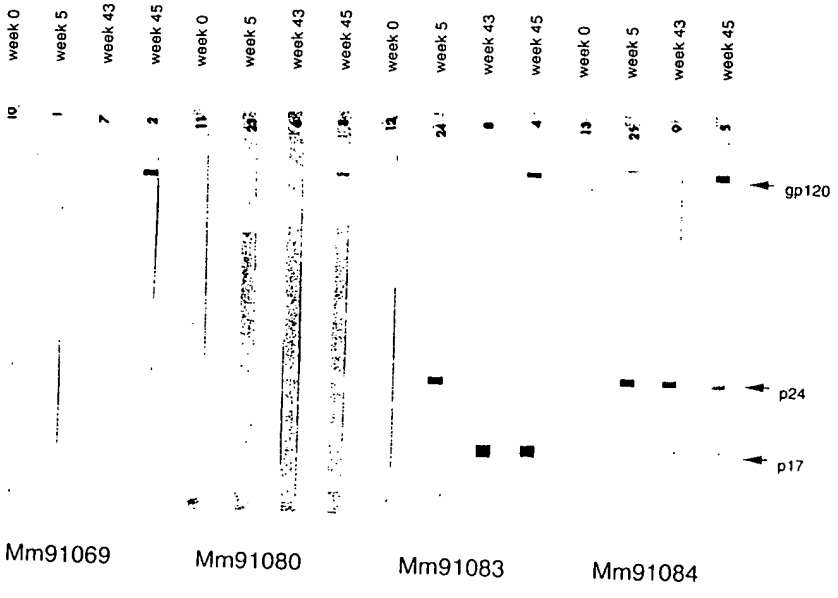

FIG. 2. Western blot analysis of serum samples from the immunized monkeys. Mm91069, 100 μg HIV-1 SF2 gp120+PCPP; Mm91080, 100 μg HIV-1 LAI whole inactivated virus; Mm91083 and Mm91084, 100 μg HIV-1 LAI whole inactivated virus+PCPP. Week 0 was preimmunization. Week 5 was 5 weeks after the first immunization. Week 43 was the week before the second immunization. Week 45 was 2 weeks after the second immunization.

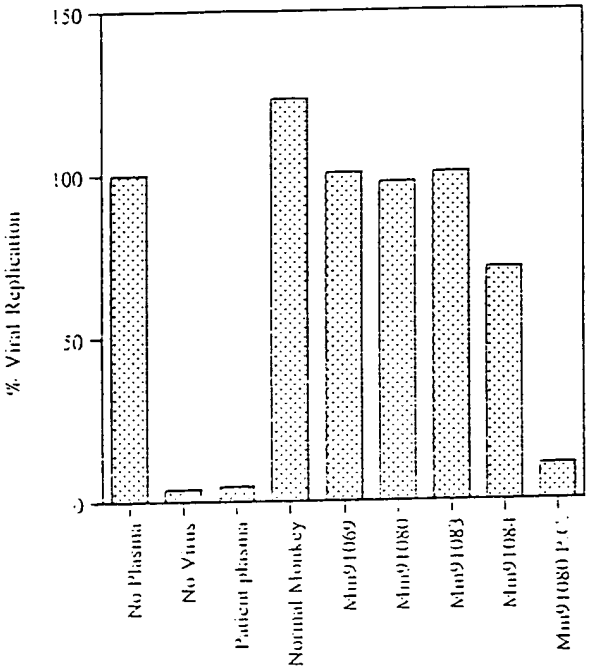

FIG. 3. Neutralization antibody analysis. The level of p27 antigen in the absence of any plasma is defined as 100% viral replication. No virus represents the background p27 level (3.8%). A HIV-1 patient plasma sample decreased the viral replication to 4.7%. A normal monkey plasma, 123.3%. The plasma from vaccinated monkeys was collected at the day before SHIV challenge. Mm91069 (100.5%), Mm91080 (97.7%), Mm91083 (100.6%), and Mm91084 (71.4%). Mm91080 P.C. (10.9%) is the plasma collected 12 weeks after the SHIV challenge.

was 32. These studies include at least five different types of antigens, 14 different protocols, and several adjuvants. It is likely that such variations in inoculum plus the limited number of animals involved in each experiment may contribute to the overall low protection rate (33%) (13). Nonetheless, the data suggests that some vaccine protection may be possible if one can use enough animals to determine optimal formulation.

TABLE 3. *Virus isolation by co-culture of PBMC from immunized and challenged monkeys*[a]

	Weeks postchallenge							
	0	2	5	8	12	16	20	24
Mm91069	—	+	+	+	+	+	—	—
Mm91080	—	—	+	—	+	+	—	—
Mm91083	—	—	—	—	—	—	—	—
Mm91084	—	—	—	—	—	—	—	—

[a] Virus isolation positive is defined as the OD value that is at least twice of that of the background. The serum samples and the negative background were measured in duplicate.

The search for an effective SIV vaccine to protect monkeys from SIV challenge has produced very important guidelines for HIV-1 vaccine development. These studies indicate a cumulative protection rate of 60% (110/179) (13). However, the fact that a human antigen present in both the vaccine formulation and the SIV challenge stocks played an important role in the protection observed in many of these studies requires careful reevaluation of the previous SIV protection data (14). A number of recent studies reported that whole inactivated SIV virus was able to stimulate protective immunity without involvement of the human antigens (15,16). However, the efficacy of inactivated vaccines appears to be very low according to a study published by the European Community Concerted Actions involving a total of 98 macaques (17).

The success of live attenuated SIV as a vaccine in rhesus monkeys suggests an alternative approach for HIV-1 vaccine development (18). A recent report indicates that the same attenuate vaccine strain behaves very differently in an infant host as compared to an adult (19). The evaluation of this approach in humans may be difficult.

The studies presented here demonstrate the potential utility of an SHIV animal model in testing the efficacy of HIV-1 vaccine candidates. Additional SHIV chimeric viruses have been constructed using envelope genes from HIV-1 ELI, HIV-1 MN, and HIV-1 89.6. Each virus stock was propagated in rhesus monkey PBMC and their $TCID_{50}$ determined. Both SHIV-MN and SHIV-89.6 replicate productively in rhesus monkeys, and SHIV-89.6 demonstrates efficient nontraumatic vaginal mucosal penetration and infection (Y. Lu and C. Miller, unpublished observation). A range of SHIV constructs titrated and characterized for growth in macaques permits the evaluation of a breadth of immune protection afforded by different antigen formulations against challenge with different iso-

TABLE 4. *Plasma viremia of challenged monkeys*[a]

	Weeks post SHIV challenge			
	0	5	12	16
Mm91069	—	200,000	800,000	800,000
Mm91080	—	800,000	200,000	200,000
Mm91083	—	—	—	—
Mm91084	—	—	—	—

[a] The numbers are the viral RNA copies in 1 ml of plasma from different times after the live SHIV-HXB challenge. The negative sign (−) means that the viral RNA copies were undetectable under the conditions used.

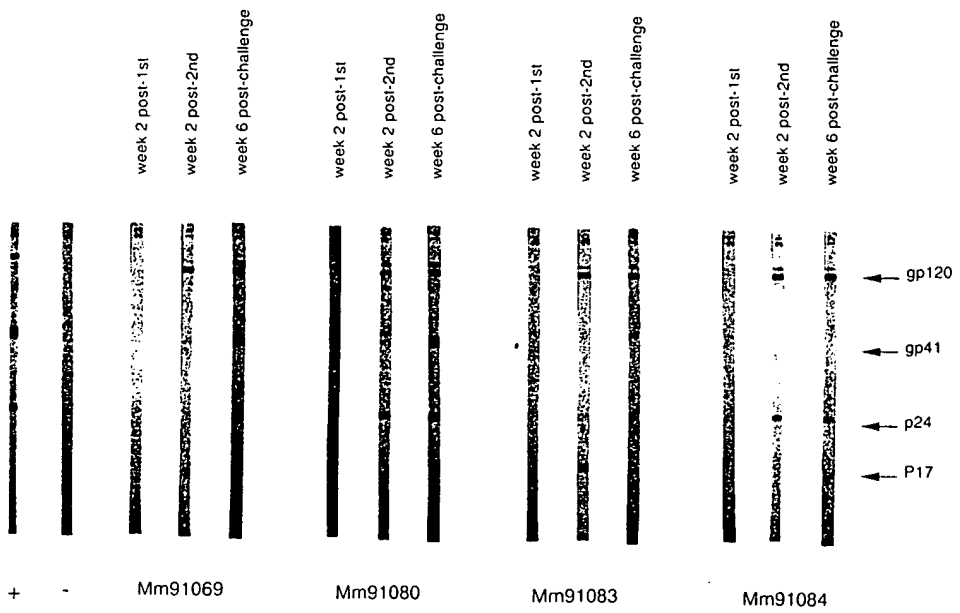

FIG. 4. Western blot analysis of serum samples from immunized monkeys before and after the live SHIV-HXB challenge. +, positive control using SHIV-infected monkeys serum; −, negative control using normal monkey serum. Week 2 post-1st, serum from 2 weeks after the first immunization; week 2 post-2nd, serum from 2 weeks after the second immunization; week 6 post-challenge, serum from 6 weeks after the challenge.

lates and by different routes of infection. Even though this study utilized a limited number of animals, it suggests that it may be possible to stimulate protective immunity in rhesus monkeys against SHIV infection by using inactivated HIV-1 plus a novel adjuvant. If this data is confirmed by a larger animal trial, one can evaluate the substitution of whole inactivated virus with different kinds of virus-like particles, or recombinant antigens that bear structural similarity to their viral counterparts and are safer to use in humans.

It is obvious that the relevance of this animal model to HIV-1 infection in humans needs to be further clarified. Given the absence of clearly defined in vitro correlates of protection against HIV-1 infection and the limited availability of chimpanzees, further exploration of the aforementioned rhesus monkey model for the preclinical evaluation of potential HIV-1 vaccine formulations is warranted.

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Exhibit 16

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
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SERIAL NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NO.
08/909,879	08/12/97	PRIEELS	J 04012.0188 02

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FINNEGAN HENDERSON FARABOW
GARRETT AND DUNNER
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WASHINGTON DC 20005-3315

EXAMINER	
BUDENS, R	
ART UNIT	PAPER NUMBER
1648	35

DATE MAILED: 02/23/99

Below is a communication from the EXAMINER in charge of this application

COMMISSIONER OF PATENTS AND TRADEMARKS

ADVISORY ACTION

☐ THE PERIOD FOR RESPONSE:

a) ☐ is extended to run _____ or continues to run _____ from the date of the final rejection

b) ☐ expires three months from the date of the final rejection or as of the mailing date of this Advisory Action, whichever is later. In no event however, will the statutory period for the response expire later than six months from the date of the final rejection.

Any extension of time must be obtained by filing a petition under 37 CFR 1.136(a), the proposed response and the appropriate fee. The date on which the response, the petition , and the fee have been filed is the date of the response and also the date for the purposes of determining the period of extension and the corresponding amount of the fee. Any extension fee pursuant to 37 CFR 1.17 will be calculated from the date of the originally set shortened statutory period for response or as set forth in b) above.

☒ Appellant's Brief is due in accordance with 37 CFR 1.192(a).

☒ Applicant's response to the final rejection, filed 1/26/99 has been considered with the following effect, but it is not deemed to place the application in condition for allowance:

1. ☐ The proposed amendments to the claim and /or specification will not be entered and the final rejection stands because:

a. ☐ There is no convincing showing under 37 CFR 1.116(b) why the proposed amendment is necessary and was not earlier presented.

b. ☐ They raise new issues that would require further consideration and/or search. (See Note).

c. ☐ They raise the issue of new matter. (See Note).

d. ☐ They are not deemed to place the application in better form for appeal by materially reducing or simplifying the issues for appeal.

e. ☐ They present additional claims without cancelling a corresponding number of finally rejected claims.

NOTE: _____

2. ☐ Newly proposed or amended claims _____ would be allowed if submitted in a separately filed amendment cancelling the non-allowable claims.

3. ☒ Upon the filing an appeal, the proposed amendment ☒ will be entered ☐ will not be entered and the status of the claims will be as follows:

Claims allowed: None
Claims objected to: None
Claims rejected: 14, 20, 23, 24, 28-31

However;

☐ Applicant's response has overcome the following rejection(s): _____

4. ☒ The affidavit, exhibit or request for reconsideration has been considered but does not overcome the rejection because the declaration does not provide convincing objective evidence that in vitro and animal data-model data reasonably correlate with in vivo efficacy in humans.

5. ☐ The affidavit or exhibit will not be considered because applicant has not shown good and sufficent reasons why it was not earlier presented.

☐ The proposed drawing correction ☐ has ☐ has not been approved by the examiner.

☒ Other Outstanding rejections are maintained for reasons of record.

Robert A. Budens

ROBERT D. BUDENS
PRIMARY EXAMINER
GROUP 1800

PTOL-303 (REV. 5-89)

Appeal Brief Due 3.26.99